



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/13, C07K 16/28, A61K 39/395, 47/48		A1	(11) International Publication Number: WO 95/20045
			(43) International Publication Date: 27 July 1995 (27.07.95)
(21) International Application Number: PCT/GB95/00118		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ). Published With international search report.	
(22) International Filing Date: 23 January 1995 (23.01.95)			
(30) Priority Data: 9401182.2 21 January 1994 (21.01.94) GB			
(71) Applicant (for all designated States except US): THE INSTITUTE OF CANCER RESEARCH: ROYAL CANCER HOSPITAL [GB/GB]; 17a Onslow Gardens, London SW7 3AL (GB).			
(72) Inventors; and (75) Inventors/Applicants (for US only): DEAN, Christopher [GB/GB]; Institute of Cancer Research, The Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB). MODJTAHEDI, Helmout [IR/GB]; Institute of Cancer Research, The Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB).			
(74) Agent: MEWBURN ELLIS; York House, 23 Kingsway, London WC2B 6HP (GB).			

(54) Title: ANTIBODIES TO EGF RECEPTOR AND THEIR ANTITUMOUR EFFECT

(57) Abstract

This invention relates to the development of agents and methods for treating tumour cells. More particularly, it is concerned with antibodies against the epidermal growth factor receptor (EGFR), which have been found to exhibit an antitumour effect, inducing terminal differentiation in tumour cells, especially those which are characterised by over-expressing EGFR. Included in the invention are these antibodies and their functional equivalents, mutants and derivatives. The antibodies may be murine (rat or mouse), but preferably have framework regions and/or constant regions based on or derived from human antibodies. The present invention also discloses that fragments of these antibodies (eg Fabs) can retain the properties of the complete antibody. The antibodies are useful, e.g. in treating or detecting tumour cells and in targeting therapies.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

ANTIBODIES TO EGF RECEPTOR AND THEIR ANTITUMOUR EFFECT**Field of the Invention**

5 This invention relates to the development of agents and methods for treating tumour cells. More particularly, it is concerned with antibodies against the epidermal growth factor receptor (EGFR), which have been found to exhibit an antitumour effect.

Background to the Invention

10 There is increasing evidence to suggest that polypeptide growth factors and their receptors are involved not only in the regulation of normal cell proliferation and differentiation but also, when aberrantly expressed, in the pathogenesis of certain types of human malignancy. The epidermal growth factor receptor (EGFR) and its ligands is one such example. This receptor is a 170kD transmembrane glycoprotein with tyrosine kinase activity which transmits the mitogenic action of the EGF family of growth factors including EGF, TGF α and amphiregulin. The binding of these ligands to the external domain of the EGF receptor initiates a number of early and delayed responses in the target cells leading ultimately to DNA synthesis and cell division.

15 Over-expression of the EGFR has been reported in a number of human malignancies including cancer of the breast, brain, bladder, head and neck, pancreas and lung (1,9-12). High levels of expression of this receptor have also been associated with poor survival in some of these patients (1,12,13). In addition, the histological and biological examination of human tumour biopsies and cell lines has shown that overexpression of the EGF receptor is often accompanied by the production of one or two of its ligands (TGF α and/or EGF) by the same tumours, suggesting that an autocrine loop may be responsible for growth of tumours of this type (14-17). Furthermore, since the ligand-induced activation of such cells acts primarily via receptors on the cell surface rather than intracellularly, such a system may form a suitable target for monoclonal antibody directed therapy (1,18-24).

20 We have described recently the production of 21 rat monoclonal antibodies raised against five distinct epitopes on the external domain of the human EGF receptor using as immunogen LICR-LON-HN5, a squamous cell carcinoma of head and neck, MDA-MB 468, a breast carcinoma cell line or A431, an epidermoid carcinoma cell line (1,25,26). Our aim was to obtain as diverse a population (isotype/epitope) of antibodies as possible from which the best mAb or combination of mAbs for clinical and diagnostic application could be selected.

25 Of these antibodies ICR64 (IgG1,) directed against epitope D and ICR16 (IgG2a) and ICR62 (IgG2b) both directed against epitope C were (in that order) the most effective at

SUBSTITUTE SHEET (RULE 26)

inhibiting ligand binding and the growth in vitro of squamous cell carcinomas overexpressing the EGFR. However, ICR62 was the most effective of the three antibodies in inducing the regression of xenografts of such tumours growing in athymic mice.

Summary of the Invention

Here we describe the results of an immunohistological examination of the events occurring in tumours during regression where athymic mice bearing established xenografts had been undergoing mAb therapy. Our aim was to investigate (a) the mechanism by which antibodies to the EGFR inhibited tumour growth and (b) to determine if viable tumour cells were present in the residual tumour nodules following treatment with antibody, and if so to determine if loss of antigen expression was a significant factor in the escape of these tumour cells.

The results of initial experiments suggested that prolonged exposure of xenografts of the HN5 tumour (a squamous cell carcinoma) which overexpresses the EGF receptor to monoclonal antibody ICR62 (IgG2b) induces a complete regression of the xenograft if antibody treatment commenced at the time of tumour implantation. Even where treatment was delayed until tumours were established, ICR62 induced complete or almost complete regression of the tumours (39).

Histological examination of the tumours remaining at the end of the experiment showed that, while few viable tumour cells could be detected, numerous keratinised areas were observed, suggesting that only differentiated tissues remained (39).

The applicants have now investigated the possibility that receptor blockade induces terminal differentiation (ie the cellular reversion to normal phenotype) of squamous cell carcinomas which overexpress the EGF receptor.

Accordingly, in a first aspect, the present invention provides antibodies to EGF receptor, and fragments thereof, for therapeutic use. Included in this aspect of the invention are those antibodies identified herein, and their functional equivalents, mutants and derivatives. The antibodies may be murine (rat or mouse), but preferably have framework regions and/or constant regions based on or derived from human antibodies. The antibodies may be complete immunoglobulin molecules, but they may be Ig fragments, for example monovalent or divalent Ig entities such as Fab fragments, single chain Fv molecules etc.

In a further aspect, the present invention discloses the use of antibodies to the EGFR, or fragments thereof, in the preparation of a medicament for inducing terminal differentiation in tumour cells, especially those which are characterised by over-expressing EGFR.

In a further aspect, the present invention provides DNA encoding the above antibodies, expression vectors comprising that DNA and host cells transformed with the expression vectors.

5 In a further aspect, the present invention provides pharmaceutical compositions comprising one or more of the above antibodies, for example selected from antibodies ICR16, ICR62 and ICR64. The amounts of the antibodies used
10 in the compositions can be determined by the skilled person, and will be typically in the range 1 to 300mg. Optionally, the the above antibodies or frgements can be conjugated to a label, toxin or drug.

15 Alternatively, the antibodies could be used to target an active agent administered in a precursor form to cells, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT (see for
20 example, EP-A-415731 and WO 90/07936).

Also included are peptide or mimetic molecules which mimic the antibody binding to EGFR and likewise induce terminal
25 differentiation of tumour cells. The present invention also includes the use of the above antibodies in the design or synthesis of these mimetics.

Although the EGF receptor antibodies mentioned above are primarily of interest in the treatment of tumour cells,
30 they may also find applications in the treatment of other disorders, eg arthritis, psoriasis, atherosclerosis, SLE, inflammation or other proliferative disorders.

Modified Antibodies

35 The antibodies described above can altered in a variety of ways using recombinant DNA technology coupled with advances in the field of monoclonal antibody and protein engineering. This has enabled access to a large selection
40 of antibodies and antibody fragments with different properties and structures to natural antibodies.

The production of monoclonal antibodies is well established in the art. Monoclonal antibodies can be subjected to the
45 techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions
50 (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma producing a monoclonal antibody may be subject to genetic mutation or
55 other changes, which may or may not alter the binding specificity of antibodies produced.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with the target, or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest (or a fragment thereof).

Antibodies may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any specific binding substance having an binding domain with the required specificity. Thus this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (eg by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

SUBSTITUTE SHEET (RULE 26)

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. 4, 446-449 (1993)), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker et al, Embo Journal, 10, 3655-3659, (1991).

Bispecific diabodies, as opposed to bispecific whole antibodies, are also particularly useful because they can be readily constructed and expressed in *E.coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

Previously, bispecific antibodies incorporating a specificity for the T-cell co-receptor CD3 have been shown to inhibit tumour growth (Titus, J. A. et al., J. Immunol. 138, 4018-4022 (1987)) and to cure lymphoma (Brissinck J. et al, J. Immunol. 174, 4019-4026 (1991)).

It may be desirable to "humanise" non-human (eg murine) antibodies to provide antibodies having the antigen binding properties of the non-human antibody, while minimising the immunogenic response of the antibodies, eg when they are used in human therapy. Thus, humanised antibodies comprise framework regions derived from human immunoglobulins (acceptor antibody) in which residues from one or more complementary determining regions (CDR's) are replaced by residues from CDR's of a non-human species (donor antibody) such as mouse, rat or rabbit antibody having the desired properties, eg specificity, affinity or capacity. Some of the framework residues of the human antibody may also be replaced by corresponding non-human residues, or by residues not present in either donor or acceptor antibodies. These modifications are made to the further refine and optimise the properties of the antibody.

Brief Description of the Drawings

Figures 1 and 2 show flow cytometric analysis of DNA in nuclei obtained from HN5 cells following four days incubation in vitro in DMEM-2%FCS alone or in DMEM-2%FCS containing anti-EGFR mAbs (156nM) or EGF (10nM). Fig 1 shows DNA histograms; Fig 2 shows the percentage of cells in each phase.

Figures 3 and 4 show the influence of antibodies to the EGFR or their Fab fragments on the binding of ^{125}I -EGF (Fig 3) or ^{125}I -TGF α (Fig 4) to the human bladder carcinoma cell line EJ.

Figures 5 and 6 show Scatchard plots of ^{125}I -EGF binding to EJ cells in the absence or presence of monovalent and divalent mAbs ICR9 (Fig 5) and ICR62 (Fig 6).

Figures 7-9 show the effect of treatment with antibodies to the EGFR or their Fab fragments on the growth in vitro of HN6 cells (Figs 7), other head and neck carcinoma cell lines overexpressing the EGF receptor (Fig 8), and the TGF α induced proliferation of quiescent human foreskin fibroblasts (Fig 9).

Figure 10 shows titration curves showing the inhibition of binding of ^{125}I -EGF to EJ cells by serum taken at the times indicated. A, patient 8 (20mg); B, patient 10 (40mg); or C, patient 13 (100mg). The starting concentration of the ICR62 standard was 10ug/ml.

Figure 11 shows the development of human anti-rat antibodies in the serum of patient number 9 following injection of 20mg ICR62 shown by binding to intact ICR62 (A), Fab ICR62 (B) and scFv ICR62 (C).

Figure 12 the development of human anti-rat antibodies in the serum of patient number 11 following injection of 40mg of ICR62 shown by binding to intact ICR62 (A), Fab ICR62 (B) but not scFv ICR62 (C).

The sequence listings show the DNA and deduced amino acid sequences of the variable regions of the heavy and light chains of ICR62 and ICR64, with the CDRs indicated in square brackets.

Detailed Description

Experiments

Part 1. Investigation of cell lines in vitro and of human tumour xenografts in vivo during or following treatment with several antibodies to the EGFR

Materials and methods

Cell lines.

The head and neck carcinoma cell line LICR-LON-HN5 and the breast carcinoma cell line MDA-MB 468 were cultured routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and the antibiotics penicillin, streptomycin and neomycin. For growth inhibition assays the concentration of FCS in medium was reduced to 2% to minimize the effects of any growth

factors present.

Monoclonal Antibodies.

5 The preparation of rat monoclonal antibodies to the extracellular domain of the human EGFR has been described previously (25,26). ICR16 (IgG2a) was raised against the receptor on the squamous cell carcinoma HN5 while mAbs ICR62 (IgG2b), ICR61 (IgG2b), and ICR64 (IgG1) were raised
10 against the receptor on the breast carcinoma cell line MDA-MB 468. Antibodies ICR16 and ICR62 bind to epitope C and antibodies ICR61 and ICR64 bind to another distinct epitope D on the external domain of the EGF receptor. Isotype matched control antibodies included ALN/11/53 (IgG2a) and 11/160 (IgG2b) which are directed against a
15 specific antigen on the rat sarcoma HSN (27), or RCI/4/74 (IgG1) an antibody directed against an idiotoxic determinant on ICR16 (unpublished data). Monoclonal antibody to cytokeratin 10 (RKSE-60) was obtained from EuroPath Ltd, Cornwall. Mouse monoclonal antibody to involucrin (28) was a gift from Dr Fiona Watt (Imperial Cancer Research Fund, London).

Treatment of mice bearing human tumour xenografts with antibodies to the EGFR.

25 Xenografts of human tumours were established in athymic (nu/nu) mice and treated with antibodies as described previously (29,39). Briefly, the protocols used were as follows.
30

A) MDA-MB 468 xenografts. Three groups of four mice were inoculated in both flanks with 5×10^6 tumour cells. On the day of tumour inoculation (day 0) one group of mice was
35 injected i.p with ICR62, the second with ICR16 and the third with control antibody. Treatment with antibody (20 μ g/dose) was continued for a further 4 consecutive days and thereafter three times weekly until day 18 (total dose 0.44mg/mouse). Animals were observed for up to 100 days
40 when the experiment was terminated and the tumour nodules remaining were excised, weighed and fixed for histological examination (see below).

B) HN5 xenografts Since treatment of athymic mice with antibody initiated at the time of tumour inoculation results in complete and permanent regression of these
45 tumours (39), HN5 tumour xenografts were set up as described above but the commencement of treatment with antibodies to the EGFR was delayed until the tumours had reached a mean diameter of about 0.5cm. In this experiment
50 groups of four to five mice were treated with antibodies ICR61 or ICR64, or with combinations of ICR61 plus ICR62 or ICR64 plus ICR62; pairs which do not compete for binding to the EGFR. In each case a second group of mice was treated
55 with control antibodies or saline. Treatment with antibodies (200 μ g/dose) was for five consecutive days and

thereafter three times weekly until the day indicated in each experiment (total dose 2.2mg/mouse). Animals were observed for up to 100 days or killed when the tumours reached a mean diameter of 0.8-1.0cm.

5

Histological examination of human tumour xenografts following treatment with antibodies.

Two protocols were used for examination of tumour specimens.

10

For routine histological examination, tumours were excised and samples were fixed in Methacarn and then embedded in paraffin. Four micron sections were cut and stained with haematoxylin and eosin (H & E).

15

All immunohistochemical studies of human tumour xenografts were performed by the indirect method using immunoperoxidase-conjugated F(ab'), rabbit anti-rat Ig as the second reagent (Star 51, Serotec Ltd, Oxford). This antibody had been preadsorbed to remove all reactivity with mouse immunoglobulins. Samples of tumour tissue were snap frozen in isopentane precooled to liquid nitrogen temperature. Sections of 5µm thickness were cut on a cryostat and mounted on glass slides that had been coated previously with 1% aminopropyltriethoxy-saline (Sigma, A3648) in acetone. Slides were dried at 37°C (30 minutes), fixed in formol-calcium for 5 minutes, and immersed in ice cold chloroform/acetone (1:1) for 5 minutes. After three washes with PBS, endogenous peroxidase was blocked by immersing the sections for 10 minutes in PBS containing 3% H₂O₂. The sections were incubated first with rat antibodies to the EGFR (1-20µg/ml, 90 minutes at room temperature) then, after three washes in PBS, with 100µl of a 1/100 dilution of immunoperoxidase conjugated F(ab'), rabbit anti-rat IgG (in PBS-0.5% BSA) for 90 minutes at room temperature. After washing twice with PBS and twice in DDW bound peroxidase was visualized using diaminobenzidine (DAB) [100mg DAB in 100ml of 0.1 M Tris buffer pH7.2, 100ml H₂O, 66µl H₂O₂]. After 5 minutes incubation, the sections were washed twice in DDW, counterstained in Mayer's haemalum for 1 minute, dehydrated by passing through graded alcohols then mounted in DPX.

20

25

30

35

40

45

In some cases, where xenografts had been treated with antibody to the EGFR, staining of the frozen sections was performed as above but without incubation in the primary antibody in order to visualize the presence of any remaining therapeutic mAb.

50

Flow cytometric analysis of tumour cells following treatment with antibody.

55

HN5 cells (7.5 x 10⁵) were seeded into 25cm² Nunc flasks (Gibco Europe Ltd, Scotland) containing 15ml DMEM plus 2% FCS. Monoclonal antibody (25µg/ml), EGF (10nM) or the

equivalent amount of medium only was then added and the cultures were incubated for four days at 37°C. Flow cytometric analysis of the nuclei prepared from these cells was performed essentially as described by Ormerod et al (30). Briefly, a suspension of single cells from each flask was prepared in 200 μ l of PBS and this was followed by the vigorous addition of 2ml ice cold 70% ethanol-30% PBS. The cells were incubated for at least 30 minutes at 4°C, harvested by centrifugation and resuspended in 700 μ l PBS. Following the addition of 100 μ l of RNase (1mg/ml, Sigma) and 200 μ l of propidium iodide (100 μ g/ml, Sigma) the suspension was incubated first at 37°C for 30 minutes and then on ice for 90 minutes. The nuclei were analysed using an Ortho Cytofluorograph 50H equipped with a Spectra-Physics argon-ion laser producing 200mW at 488nm and an Ortho 2150 computer system (30).

Immunofluorescent staining of cytokeratin 10 and involucrin.

5x10⁴ HN5 cells were plated onto glass cover slips in 24 well plates containing 1ml DMEM plus 2%FCS. Following overnight incubation at 37°C, specific or control antibodies (25 μ g/ml) or medium alone were added to the cultures and the cells were incubated for a further three to four days at 37°C. After two washes with PBS, the cells were fixed for 5 minutes in ice-cold methanol then washed by incubation in PBS for 30 minutes. Mouse anti-cytokeratin 10 or anti-involucrin mAb diluted in PBS-0.5% BSA was added and the coverslips were incubated at 4°C for 1 hour. After three washes, bound primary antibody was detected using fluorescein-conjugated sheep anti-mouse Ig (Amersham International). The coverslips were mounted in Hydromount: glycerol (1:1) and examined for green fluorescence using a Zeiss Axiovert 100 microscope.

Results

Histological examination of regressing tumours.

H & E staining

We have shown previously that when athymic mice bearing xenografts of the breast carcinoma MDA-MB 468 were treated from day 0-18 with a total dose of 440 μ g of ICR16 (IgG2a), half of the tumours regressed completely but small static nodules persisted at the remaining sites when the experiment was terminated on day 100 (39). However, the present experiments showed that similar treatment with mAb ICR62 (IgG2b) resulted in complete eradication of all tumours. Histological examination of H&E stained sections of the tumour nodules remaining following treatment with ICR16 showed that a few areas contained apparently viable tumour cells amongst the largely necrotic zones. The necrotic cells also showed substantial loss of cytoplasmic staining following treatment with ICR16 compared with the

progressively growing tumours treated with control antibody.

5 We have also examined HN5 tumour xenografts following treatment with one, or a combination of two anti-EGFR mAbs that bind to two distinct epitopes on the external domain of the human EGF receptor. When athymic mice bearing HN5 xenografts were treated from day 7-24 with a combination of ICR62 plus ICR64 (total dose 2.2mg/mouse), on termination 10 of the experiment (day 79) complete control was observed at 2/10 sites and the tumour nodules remaining were still undergoing regression (29). Histological examination of these tumour nodules showed the presence of few if any viable cells and the lesions were composed largely of scar 15 tissues compared to tumours that were growing in animals treated with the control antibody.

20 Similarly, when HN5 tumour xenografts were treated from day 5-24 with antibody ICR61, 2/8 tumours had disappeared completely when the experiment was terminated on day 75 (29). Examination of the nodules remaining also showed that few if any viable cells were present and the lesions were similar in appearance to those described above.

25 An H & E stained section of an HN5 nodule remaining after treatment from day 7-24 with a combination of ICR61 plus ICR62 (total dose 2.2mg/mouse) showed that zones of keratinisation and apparent squamous differentiation were present. Indeed, keratinized areas could be found in all of 30 the tumour lesions remaining in mice that had undergone treatment with the specific antibodies used either alone or in combination. These findings suggest that squamous differentiation of HN5 was an important consequence of treatment with EGFR-specific antibodies.

35 Immunoperoxidase staining

40 An integral part of this study was to determine if any viable tumour cells remained after treatment with anti-EGFR antibody that continued to express high levels of the EGFR or if loss of this antigen could contribute to their escape. While none of the rat mAbs used in this study bind to the EGFR in formalin-fixed paraffin embedded sections, they are all effective in staining the membranes 45 of cryopreserved tissues. Strong membrane reactivity was obtained when frozen sections of HN5 tumour xenografts were stained with specific antibody. No staining was obtained if treatment with specific antibody was omitted or when the sections were pretreated with control antibody.

50 We have shown previously that when athymic mice bearing xenografts of the HN5, A431 or MDA-MB 468 tumours were treated with antibody ICR62, the tumours regressed more rapidly, (and in most cases completely), compared with the 55 same tumours treated with mAbs ICR16 or ICR64 (39). We have carried out an immunohistochemical investigation of

regressing tumours to determine the reasons for the greater efficiency of antibody ICR62 in vivo. First, athymic mice bearing HN5 xenografts were treated for a short period (days 0-4) with either ICR16 or ICR62. On day 7 tumours were excised, cryosections were prepared and stained with peroxidase conjugated F(ab'), rabbit anti-rat IgG. The uniform staining of the tumour cell membranes with the second antibody, shows that all of the tumour cells were coated with specific rat antibody at this time. The total area of tumour remaining was smaller in the mice treated with ICR62 (mean tumour diameter 27.5% of control) however, compared with those treated with ICR16 (mean tumour diameter 86% of control). Also, the ICR62 treated tumours showed a more extensive host cell infiltrate surrounding the remaining viable tumour cells.

Finally, we have examined the residual nodules at day 101 after mice bearing established xenografts of the HN5 tumour were treated with ICR64 from day 7-24. Despite the fact that the last treatment with antibody was given 77 days previously, staining of the sections with peroxidase conjugated anti-rat Ig showed that significant amounts of rat mAb remained associated with the dead cells and the keratinized areas where tumour destruction was complete. Since the second antibody used was a F(ab'), preparation that had been pre-adsorbed against mouse Ig the staining could not have been due to non-specific binding to mouse Ig or Fc-receptor bearing cells. In contrast, the small areas of viable tumour did not stain, suggesting either that ICR64 had not reached these locations or that it had been lost from the cell membranes during cell proliferation. To determine if these cells still overexpressed the EGFR, consecutive sections were treated first with ICR64 and then with the peroxidase conjugated second antibody. Here, the nests of viable cells stained positively suggesting that loss of antigen was not a significant factor in the escape of these cells from antibody treatment.

HN5 cells treated with antibodies to the EGFR undergo terminal differentiation.

The finding of keratin whorls in tumour nodules remaining after antibody treatment was of particular interest. We have shown previously that, at concentrations above 5nM, mAbs ICR16, ICR62 and ICR64 inhibit completely the growth of HN5 cells cultured in medium containing 2% FCS (25,26). To investigate the possibility that terminal differentiation could be a pathway for tumour cell inactivation we have determined the cell cycle characteristics of growth arrested cells and examined them for expression of the differentiation markers involucrin (31-33) and cytokeratin 10 (34,35).

Growth arrested HN5 cells accumulate in G0/G1.

Flow cytometric analysis of nuclei following treatment of

HN5 cells with either antibodies to the EGFR (156nM ICR16 or ICR62) or with the ligand EGF (10nM) is illustrated in Figures 1 and 2. After treatment with doses of anti-EGFR mAbs which inhibited completely the growth of HN5 cells, the numbers of cells in S and G2/M had decreased substantially compared with the controls growing in medium alone, and most of the cells were arrested in G0/G1. Treatment with EGF at a concentration (10nM) which also inhibited (but not completely) the growth of HN5 cells, also resulted in a decrease in the percentage of cells in both S and G2/M phases of cell cycle but to a lesser extent than following treatment with the mAbs. We did not see any evidence for DNA fragmentation in a pre-G1 peak indicative of apoptosis (figure 1).

Growth arrested HN5 cells synthesize markers of terminal differentiation.

When HN5 cells were incubated for four days with 156nM of the anti-EGFR mAbs most of the cells were found to express the differentiation marker cytokeratin 10 as visualised by immunofluorescence with mAb RKSE-60. The proportion of positive cells varied with the anti-EGFR antibody used, the most effective being ICR64, and by day 4 the majority of the treated cells were strongly positive for this differentiation marker. In addition the majority of cells expressing cytokeratin 10 were larger than the cytokeratin negative cells. Similar results were obtained when the treated HN5 cells were stained with antibody to another differentiation marker, involucrin.

Essentially identical results were obtained when HN5 cells were treated with ICR16 or ICR62 and examined for CK10 and involucrin expression (data not shown). However, neither differentiation marker was expressed by cells that had been grown to near confluence in medium alone or with control antibody although these cultures contained approximately ten times more cells than in the wells treated with antibodies to the EGFR.

Part 2 Further investigation of the mechanism of action of the antibodies and their Fab fragments

Antibody ICR62, which binds to epitope C on the EGFR inhibits (a) the binding of ligand to the receptor, (b) the EGF and TGF α induced proliferation of human fibroblasts and (c) the growth of a range of human tumour cells that overexpress the EGFR (26). Antibody ICR9 binds to epitope A on the EGF receptor, and (a) increases the binding of ligand (EGF and TGF α) to the EGF receptor, (b) enhances the EGF induced proliferation of human fibroblasts, and (c) stimulates the growth of tumours over-expressing the EGF receptor (25). Here, our aim was to discover if the monovalent Fab fragments of these mAbs were as effective as the bivalent mAbs in inducing these effects or if bivalent antibody was essential for their activity

(2,3,5,7,44,45).

Methods

5 Referring to Figures 3 and 4; monoclonal antibodies ICR9 and ICR62, and their Fab fragments, were prepared as described previously (25,26,36). Fab fragments of ICR9 and ICR62 were prepared by papain digestion and purified by gel and affinity chromatography. Analysis by SDS-PAGE indicated
10 that the Fab preparations were free of contaminating intact antibody (data not shown). Human recombinant EGF and TGF α (Collaborative Research, Waltham, Mass) were labelled with Iodine-125 (Na¹²⁵I, Amersham International) using IODO-GEN, to a specific activity of 10 μ Ci/ μ g as described previously
15 (25). A competitive RIA was used to determine the effect of the mAbs and their Fab fragments on the binding of ¹²⁵I-EGF and ¹²⁵I-TGF α to the EGFR on EJ cells as described (26). The rat mAbs 11/160 and ALN/11/53 directed against an irrelevant antigen (27) were used as controls.

20 Referring to Figs 5 and 6; ¹²⁵I-EGF was prepared as described above. EJ cells were seeded into 96 well plates (2x10⁴ cells/well) in DMEM containing 10% FCS. Following 48 hours incubation at 37°C, the cells were washed twice with ice cold binding buffer (DMEM, 15mM Hepes containing 0.1% BSA)
25 and incubated with 20 μ l of ICR9, Fab ICR9, ICR62 or Fab ICR62 (20 μ g/ml) or medium alone for 30 minutes on ice prior to addition of 30 μ l of ¹²⁵I-EGF (0.86-104nM). Following a further 5 hours incubation on ice, the cells were washed three times with binding buffer, lysed and the bound radioactivity was determined. Non-specific binding was
30 corrected by subtracting the counts bound in the presence of a 100-fold excess of cold EGF and was less than 6% of the total counts bound. Each value is the mean of triplicate samples.

Referring to Figures 7 and 8; HSC-1, HSC-2, HSC-4, Ca9-22, LICR-LON-HN5 and LICR-LON-HN6 cell lines (6,9) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented
40 with 10% foetal calf serum (FCS) and the antibiotics penicillin, streptomycin and neomycin. To investigate the effect of treatment with intact mAbs or their Fab fragments on the growth of the tumour cells in vitro about 5x10³ cells in 100 μ l of DMEM containing 2% FCS were seeded into each well of a 96-well plate. After incubation for 4 h at 37°C,
45 100 μ l aliquots of dilutions of mAbs or Fab fragments were added to triplicate wells and the cultures were incubated at 37°C. When the cells in the control wells (containing medium alone) were nearly confluent, all cells were fixed, stained with methylene blue and the A₆₂₀ determined as
50 described previously (26).

Referring to Figure 9; DE532 cells (Flow Laboratories) seeded at 4 x 10⁴ cells/ml in EMEM-10% FCS into 24-well
55 plates were grown to confluence then the medium was replaced with EMEM-1%FCS. After 48 hours in this medium,

SUBSTITUTE SHEET (RULE 26)

50 μ l aliquots of mAb or Fab fragment (25 μ g/ml) and/or TGF α (5ng/ml) were added to triplicate wells and the cells were incubated overnight at 37°C then pulsed for 6 hours with 2 μ Ci/well of ³H-thymidine. The acid insoluble radioactivity incorporated into DNA was determined in a liquid scintillation counter.

Photomicrographs of HN5 tumour cells were taken following treatment for four days with 156nM of ICR62, ICR62 Fab fragment or control antibody 11/160 (27), and viewed alternatively by phase contrast illumination or by immunofluorescent staining for expression of cytokeratin 10.

5 x 10⁴ HN5 cells were plated onto glass cover slips and placed in 24 well plates containing 1ml DMEM-2% FCS/well. Following overnight incubation at 37°C, specific or control antibodies (25 μ g/ml) were added to the cultures and the cells were incubated for a further four days at 37°C. After two washes with PBS, the cells were fixed for 5 minutes in ice-cold methanol then washed by incubation in PBS for 30 minutes. Mouse anti-cytokeratin 10 (RKSE-60, EuroPath Ltd, Cornwall) diluted 1/40 in PBS-0.5% BSA was added and the coverslips were incubated at 4°C for 1 hour. After three washes, bound primary antibody was detected using fluorescein-conjugated sheep anti-mouse Ig (Amersham International Ltd). The coverslips were mounted in Hydromount:glycerol (1:1) and examined for green fluorescence using a Zeiss Axiovert 100 microscope.

Results

When the Fab preparations of ICR9 and ICR62 were tested for their effects on ligand binding they were found to enhance (ICR9) or inhibit (ICR62) the binding of both ¹²⁵I-EGF and ¹²⁵I-TGF α to the receptor on the bladder carcinoma cell line EJ with similar efficiencies to the bivalent parental IgGs. At the same concentration, a control rat antibody (ALN/11/53), directed against an unrelated antigen (27) was without effect on the binding of ligand. Scatchard analysis of these data showed that treatment of EJ cells at 4°C with monovalent or bivalent ICR9 promoted the binding of ¹²⁵I-EGF to the receptor by increasing the affinity of the receptor for ligand (Figure 5). The K_d for binding of ¹²⁵I-EGF was 2.1 x 10⁸ M⁻¹ in medium alone and 7 x 10⁸ M⁻¹ or 1.8 x 10⁹ M⁻¹ in the presence of bivalent or monovalent ICR9 respectively. On the other hand, monovalent and bivalent ICR62 inhibited the binding of EGF completely (Figure 6).

We then compared the monovalent Fab fragments with the parental bivalent antibodies for their effects on the growth of the head and neck carcinoma cell line HN6, which expresses about 1.2 x 10⁶ EGFR/cell (26). The results illustrated in Figure 7 show that at concentrations above 0.6nM both the Fab fragment and intact ICR9 stimulated the growth of HN6 cells. The Fab fragment of ICR62, likewise,

was found (Figure 7) to be as effective as the bivalent mAb at inhibiting the growth in vitro of HN6 cells. Moreover, the monovalent ICR62 inhibited the growth of five other head and neck tumour cell lines (HN5, HSC-1, HSC-2, HSC-4 and Ca9-22) (6,9) which overexpress the EGF receptor (figure 8). Like the intact ICR62, monovalent Fab fragments of this antibody also inhibited completely the TGF α induced proliferation of quiescent human foreskin fibroblasts (Figure 9). Furthermore, the Fab of ICR62 was as effective as the bivalent antibody in inducing the differentiation of HN5 cells. The finding that Fab fragments of the rat mAbs ICR9 and ICR62 were as effective as the intact molecule in inducing the respective changes in the function of the EGF receptor was unexpected since other investigators had shown that Fab fragments of murine antibodies were either poorly active or ineffective compared to the intact antibody (3,5,7,8,44,45).

Discussion

The results of our studies (Part 1 above) have shown that the nodules remaining at up to 82 days following the last treatment with antibodies to the EGF receptor consisted largely of necrotic and keratinized areas with few viable cells present. In another study (37) it was reported that treatment of an established human colorectal tumour xenograft with high doses of the mouse anti-EGFR mAb 225 (2mg twice weekly) resulted in complete regression of these tumours by 2-3 weeks (37). This study also showed that by day 7 most of the cells were necrotic and after 14-21 days most tumour cells had been replaced by connective tissues (37). In the present investigation, the presence of keratinized areas was of particular interest and this finding pointed to a previously undescribed effect of treatment with antibodies to the EGFR; namely that prolonged receptor blockade can induce terminal differentiation of the squamous carcinoma cells. We have investigated this function of antibodies to the EGFR by screening the anti-EGFR mAb treated cells for expression of the terminal differentiation markers involucrin, a 92 kD cytoplasmic precursor of cornified envelope (31-33) and cytokeratin 10 expressed during terminal differentiation in squamous epithelia (34-35).

We have shown that during treatment with antibodies to the EGFR the HN5 cells were undergoing terminal differentiation and the majority of the treated cells expressed the differentiation markers involucrin and cytokeratin 10. Furthermore, we also noted that cells expressing these differentiation markers were of larger size as previously shown for cultured human keratinocytes (32). The results of our flow cytometric analysis of HN5 cells following treatment with anti-EGFR mAbs showed that, as would be anticipated for differentiated cells, they were arrested in the G0/G1 phases of the cell cycle. Taken together these results suggest, for the first time to our knowledge, that

antibodies against the EGF receptor which block growth factor-receptor interaction, may inhibit the growth of EGFR overexpressing tumours by inducing differentiation. In agreement with our findings, Rodeck and colleagues have shown that growth inhibition of A431 cells by a mouse antibody to the EGFR (mAb 425), results in a decrease in the percentage of cells in S and G2/M phases of the cell cycle and a increase in the percentage of cells in G0/G1 phases of the cell cycle (20). A similar mode of action was also suggested recently by Bacus and colleagues (38) for the effect of growth inhibitory antibodies directed against the external domain of the HER-2/c-erbB-2 receptor, which like EGFR belongs to the type I growth factor receptor family (4). This study also provided evidence for a relationship between the in vivo antitumour activity of anti-HER-2/c-erbB-2 mAbs and their capacity to induce differentiation of breast cancer cells in vitro (38).

Two other interesting findings came from the immunohistochemical staining of the tumour specimens. Firstly, it was clear that the increased rate of tumour regression seen during treatment with ICR62 (IgG2b) was due to rapid loss of tumour cells and, in addition, more infiltrating host cells were observed surrounding the tumour foci. As discussed above, although not the most effective inhibitor of growth in vitro, antibody ICR62 was the most effective inducer of the regression of three different human carcinomas grown as xenografts in athymic mice (39). These results point to a role for host immune effector functions in vivo. Indeed, it is well documented that rat antibodies of the IgG2b isotype, like murine IgG2a and human IgG1, are the most effective at mediating ADCC with Fc-receptor bearing effector cells and in activating the complement cascade (20,40). Secondly, we were surprised to find that in the nodules remaining following treatment of the HN5 tumour with ICR64, cell membranes in the necrotic areas still retained antibody that had been given 77 days earlier. This unexpected finding points to the stability of the immune complexes formed and is consistent with the results of experiments in vitro which indicate that the immune complexes formed on binding of these antibodies to HN5 cells are stable and are not rapidly internalised or shed from the cells. The small number of viable cells remaining, which did not stain with the anti-rat Ig reagent, had not lost their expression of the EGFR since they were positive when restained using ICR64 as the first reagent. We conclude that antigenic modulation was not a significant factor in the escape of viable cells from antibody treatment and, providing antibody access was satisfactory, these cells might be susceptible to further treatment with antibody.

Conclusions.

On the basis of our data we conclude that the best antibodies for clinical application will be those which are

directed at the correct epitope and are also the most effective at inducing terminal differentiation and recruiting and activating host immune effector functions (43).

5 The results of the further experiments (Part 2 above) showed that, after four days incubation in the presence of 156nM monovalent or bivalent ICR62 the majority of HN5 cells expressed the terminal differentiation marker
10 cytokeratin 10 (34). Control cultures grown in the absence of specific antibody did not express cytokeratin 10, although by four days the cell monolayers were almost confluent.

15 The finding that blockade of the EGF receptor by ICR62 Fab can induce the terminal differentiation of HN5 cells is of some interest and may have clinical application. To our knowledge this is the first report to show that smaller molecules such as the monovalent Fab fragment of an
20 anti-EGFR antibody can be as effective as bivalent antibody in (a) blocking ligand binding, (b) preventing the growth of tumours overexpressing the EGF receptor, (c) directing terminal differentiation in such cells. The possibility that smaller molecules than intact antibody can be employed
25 to induce these effects could be significant for clinical application since the rate of extravasation of the smaller molecules should improve uptake into tumour, although clearance from the blood will be increased.

30 The mechanism by which EGF activates the EGF receptor has not yet been resolved and two models (intramolecular or intermolecular activation) have been proposed. The intramolecular model suggests that binding of EGF to the EGF receptor induces a conformational change in the
35 extracellular domain of a receptor monomer which is propagated across the plasma membrane to the cytoplasmic kinase domain leading to its activation (46,49). The intermolecular model (2,3,48) on the other hand, proposes that receptor dimerization following ligand binding
40 triggers phosphorylation by the cytoplasmic tyrosine kinase. On the basis of the second model it was expected that bivalent antibody would block activation by preventing receptor dimerization (3,7,8,44). However, the results of a recent investigation using antibody 13A9 (47), whose
45 activity has been shown to take place at a distance from the EGF binding site suggest that this is not the case. In this study, Carraway and Cerione (47) have shown that treatment of A431 cells with this antibody prevents EGF receptor aggregation following binding of EGF but does not
50 prevent activation via the receptor as judged by several parameters including receptor autophosphorylation, EGF-stimulated changes in cytosolic free $[Ca^{2+}]$ and mitogenesis. They provide convincing evidence to suggest that conformational changes in the receptor, not receptor
55 aggregation, is the signal generated following ligand binding.

SUBSTITUTE SHEET (RULE 26)

Our results would support this view. With bivalent and monovalent ICR9, it is clear that binding of this antibody causes a marked increase (three to nine-fold) in the affinity of the receptors for EGF. Since these experiments were carried out at 4°C it is unlikely that this result could be due receptor aggregation. Furthermore, because ICR9 competes with neither the EGFR ligands nor the antibodies that block ligand binding we conclude that it acts at a distance i.e. it induces a conformational change at the ligand-binding site. On this basis we propose that, some at least of the antibodies which prevent ligand binding do so by altering the conformation of the receptor to reduce its affinity for ligand. This concept is leant support by the observation that only some of the rat antibodies which block binding of ligand to the EGF receptor are able to displace bound ligand i.e some of the antibodies must act at a distance from the ligand binding site.

The possibility that scFv or constrained peptides based on the complementarity determining regions of immunoglobulins can be developed for effectively targeting the EGFR on squamous cell carcinomas is an exciting prospect especially if such drugs can induce terminal differentiation of the tumour cells. The possibility also exists for developing non-peptide mimetic molecules, which mimic the action of these binding proteins or peptides, and have a similarly effective interaction with the EGFR. The anti-EGFR antibodies herein can be used to screen for such non-antibody molecules which have a similar effect on tumour cells.

The accompanying sequence listings show within square brackets the complementary determining regions (CDRs) for the heavy and light chains of antibodies ICR62 and ICR64, together with their surrounding framework regions (FRs). These CDRs can be grafted into the FRs of human antibodies, optionally with retention of selected murine residues in the FRs, according to well established principles discussed above (see for example, Winter GB-A-2188638, Harris et al WO92/04381 and Queen et al, PNAS (1989), 86:10029-33) to create corresponding humanised antibodies which may be more appropriate for the treatment of humans.

Clinical Study Using ICR62 Monoclonal Antibody

For clinical application, it is important to recognise that the EGF receptor is also expressed on some normal human tissues but the level of expression is much lower than on the corresponding tumour cells (9-11). However, in our experience tumours which express 10^5 or less receptors are much less sensitive to antibody treatment than are the EGFR overexpressing tumour cells (1,26). Indeed, Mendelsohn and colleagues in a preclinical study with chimpanzees, have reported that treatment with a total dose of 650mg of antibody produced no toxicity (41). In addition, in a

phase I clinical trial where patients with lung carcinoma were treated with doses of up to 300mg of the mouse antibody 225 they observed no untoward effects in the patients (41,42).

mAb ICR62 was then tested in a Phase I clinical study in patients with cancer of the head and neck (11 pts) or Lung (9 pts) cancers. A majority of the patients in this study had undergone previous treatment (i.e, surgery, radiotherapy, chemotherapy, and/or immunotherapy). The aims of this clinical study were to (a) monitor patients for any possible signs of toxicity, (b) investigate if therapeutic antibody had localised specifically to tumour cells in metastatic lesions, (c) determine the level of ICR62 remaining in circulation following treatment and (d) discover if the patients mounted a human anti-rat antibody (HARA) response.

Materials and Methods

Patient Selection and Treatment

Patients were considered eligible for inclusion in this trial who had i) inoperable histologically or cytologically confirmed diagnosis of squamous cell carcinoma of the lung or head and neck, ii) immunocytochemically proven expression of the EGF receptor, iii) an ECOG performance status of 0-2, iv) no known history of allergy or atopy, v) no immunological therapy within the previous 4 weeks, vi) no significant abnormalities of renal, hepatic, or bone marrow function (haemoglobin > 10g/dl, white count > $3 \times 10^9/l$, platelet > 120, creatinine < 130, liver enzymes and bilirubin < x2 normal).

Details of tumour grade and the previous treatments that the patients had received are summarised in Table 1. Patients were skin tested (10ug intradermally) 1hr before antibody was administered and no patient was found to give an adverse response. Antibody in phosphate buffered saline pH 7.4 was given intravenously as a single bolus injection over a period of 30-60 minutes. Groups of three patients were treated with 2.5, 10, 20, or 40mg of ICR62 and a further eight patients were given 100mg of this antibody. All patients were evaluated for signs of toxicity. Also, blood samples were taken from these patients before and at intervals after treatment with ICR62 so that the serum could be monitored for the level of mAb ICR62 in circulation and tested for the presence of human anti-rat antibodies (HARA). In some of the patients, given antibody doses of 40mg or 100mg, biopsies were taken from accessible metastatic lesions, 24 hours following treatment with ICR62 and examined for the localization of ICR62 to the tumour cells.

Preparation of mAb ICR62

All antibody for clinical use was prepared according to the guidelines prepared by the CRC-MRC Joint Committee. Hybridoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 3% or 5% fetal calf serum of North American origin and antibiotics as described previously (27), in either in a Verax Type 1 Bioreactor or as bulk cultures in roller bottles. Supernatants were harvested under aseptic conditions and then precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 45% saturation. Using autoclaved reagents, column packings and containers, the precipitates were dissolved in water and dialysed against 0.0175M phosphate buffer pH 6.6. After centrifugation in a Beckman 45Ti rotor at 30,000g to remove insoluble material, the dialysate was fractionated by passage through a column of Whatman DE52 cellulose equilibrated with and eluted with 0.0175M phosphate buffer pH 6.6. The flow through fractions containing the purified (>95% mAb) ICR62 were bulked and dialysed against five changes of sterile phosphate buffered saline (PBS). After filter sterilisation the preparation was aliquoted, frozen and stored at -20°C until use.

Determination of the level of ICR62 in Circulation

The amount of free mAb ICR62 present in serum was determined by its ability to inhibit the binding of EGF or TGF to the bladder carcinoma cell line EJ as described previously (27). Doubling dilutions of serum ($50\mu\text{l}$) were mixed with an equal volume of ^{125}I -EGF (4×10^4 cpm) or ^{125}I -TGF (4×10^4 cpm). Standards containing known concentrations of ICR62 were set up in the same way. Aliquots of $90\mu\text{l}$ of each mixture were then transferred to monolayers of EJ cells grown to confluency in 96 well plates. After incubation for one hour on ice the cells were washed three times, then lysed in 1M NaOH containing 1% sarkosyl and the bound radioactivity was determined in a Hydragamma spectrometer (Oakfield Instruments Ltd., Oxford).

Determination of Human Anti-Rat (HARA) Response

Polyvinyl chloride 96-well plates (Dynatech Labs, Virginia), were coated with rat antibody by incubation overnight at 4°C with $50\mu\text{l}$ /well of a stock solution ($10\mu\text{g}/5\text{ml}$ of PBS) of ICR62, ICR62 Fab, or ICR62 scFv. The plates were washed three times with PBS containing 0.5% BSA and then incubated for 2 hours with $200\mu\text{l}$ /well of PBS-0.5% BSA to block the remaining sites. After a further three washes with PBS containing 0.5% BSA, doubling dilutions of the patients sera made in PBS-0.5% BSA were added in duplicate to the wells and the plates were incubated for 1 hour at ambient temperature. After washing the plates three times with PBS-0.5% BSA, human antibodies bound to ICR62 or its fragments were detected by the addition of ^{125}I -labelled rabbit anti-human F(ab')₂. After incubation

for 1 hour at ambient temperature, the plates were washed three times with PBS-0.5% BSA and then cut into individual wells and the bound radioactivity determined in a Hydragamma counter.

5

Immunohistochemistry

All immunohistochemical studies were performed by the indirect method using sheep antibodies to rat F(ab')₂ conjugated to horseradish peroxidase (Amersham International).

To determine expression of the EGFR, tumour biopsies were snap frozen in liquid nitrogen then mounted in OCT medium and sections of 5µm thickness cut. One section was stained with Haematoxylin & Eosin (H&E) to determine the nature of the tumour sample. A second sample was fixed in acetone at 4°C for 10 minutes, then after washing briefly in PBS the section was incubated with mAb ICR62 (100µg/ml, for 1 hour. Following washing in PBS for 5 minutes, the sections were incubated with a 1:100 dilution of sheep anti-rat F(ab')₂ conjugated to horse radish peroxidase (Amersham) for 45 minutes. Peroxidase staining was demonstrated by incubating the sections for 10 minutes in a solution containing 0.05% diaminobenzidine (Sigma), 0.1% hydrogen peroxide (Merck) and 0.07% imidazole (Merck). After washing in running tap water for 5 minutes, the sections were counterstained in Mayer's Haematoxylin (HD Suppliers) for 30 seconds. Finally the sections were dehydrated, cleared and mounted.

To monitor the localisation of ICR62 to tumour cells in patients that were given doses of 40mg or 100mg of antibody, biopsies were taken from metastatic sites, 24 hours after the antibody was administered, then frozen sections were cut and stained with sheep anti-rat antibody alone. To determine the proportion of cells that had bound therapeutic antibody, serial sections taken from the same biopsies were stained first with ICR62 followed by the peroxidase conjugated sheep anti rat F(ab')₂ so that all EGFR expressing cells were stained.

Results.

Effect of treatment on patients

Twenty patients with squamous cell carcinoma of the head and neck or lung whose tumours were found to overexpress the EGF receptor were recruited into this dose escalation trial to assess the toxicity of the rat mAb ICR62 (Table 1). All had extensive disease and, with the exception of four patients (see Table 1), had received previous treatment(s) for their disease e.g. surgery, radiotherapy and/or chemotherapy (Table 1).

55

Patients were admitted to hospital on the day before the

administration of antibody and their biochemical and haematological parameters were monitored. Following intravenous injection of the antibody some of the patients exhibited mild rigors and fever or hypotension (Table 1). The symptoms which were not related to the dose of antibody administered were readily controlled. In no case were any severe toxicities observed. The patients were released from hospital 24 hours after the injection of antibody and they were seen at weekly intervals for up to six weeks. None of the patients reported any untoward effects of their treatment during the follow-up period.

Blood levels of ICR62

We have investigated if mAb ICR62 could be detected in the serum of patients given different doses of this antibody by monitoring the inhibition of binding of EGF or TGF to the EGFR by sera taken at intervals following treatment. We have been unable to detect any free antibody in the serum, taken at 4 hours or 24 hours post-treatment, of patients that were given 20mg or less of ICR62 (Figure 1a). However, significant amounts of circulating ICR62 could be detected, in the blood of patients given doses of 40mg or 100mg of ICR62 (Figure 1b & 1c). In addition, the level of ICR62 remaining in circulation was found to be highest in patients receiving 100mg of ICR62. In one patient, about half of the antibody administered was in circulation on day 3 but no free mAb could be detected in the blood at 7 days post ICR62 treatment.

Injected ICR62 binds to the EGFR on tumour cell membranes

Biopsies were taken of metastatic lesions from six patients, 24 hours after treatment of the patients with 40mg or 100mg of ICR62. Frozen sections were examined for the presence of cell bound antibody by staining directly with a peroxidase-conjugated second antibody reagent. Two of the biopsies were found to consist largely of necrotic material and were discarded but four yielded well defined regions containing infiltrating tumour. The sections illustrated in figure 2a show that 24 hours after treatment of two patients with 40mg of ICR62, the antibody had localized to the membranes of tumour cells in the metastatic sites and the proliferating cells at the periphery of the tumour were strongly stained. When a sequential section was stained with ICR62 prior to the addition of the sheep anti-rat reagent, it was found that cells at the interior of the tumour which also expressed the EGFR had not bound the therapeutic antibody administered 24 hours earlier (figure 2b). However, when frozen sections of biopsies obtained from metastases of two patients treated with 100mg of ICR62 were examined, it was clear that the mAb had penetrated further into the metastatic lesions (Figures 3a & 3b). Again, very good membrane staining of the proliferating cells at the tumour periphery was observed.

Development of human antibodies to ICR62

We have investigated the immunogenicity of mAb ICR62 in these patients by determining if human anti-rat antibodies (HARA) were present in the serum of patients given different doses of ICR62. Also, we have investigated if the human anti-rat response included anti-idiotypic antibodies by determining if the antibodies in the patients serum bound to scFv fragments of ICR62. Of the twenty patients treated with ICR62, human anti-rat antibodies were detected in the blood of only four patients (numbers 9, 10, 16 and 19 see Table 1).

Of these only two patients given 20mg (No 9) or 40mg (No 11) produced anti-idiotypic antibodies which bound to scFv ICR62 (Table 1, Figure 4). Sera from two out of the eight patients treated with 100mg of ICR62 (patients 16 and 19) contained antibodies directed against determinants on the constant region since they bound to the Fab and intact antibody but not to the ICR62 scFv (Table 1, Figure 5). In one patient (No 12), the results obtained with the serum taken before treatment indicated that antibodies which bound to rat IgG were present in the sera before ICR62 was administered but tests were not done to determine if binding was specific or due to the presence of autoantibodies such as rheumatoid factor.

Discussion.

Overexpression of the EGFR accompanied by production of the EGF family of ligands has been found to occur in a wide range of human malignancies and this phenomenon has been correlated with a poorer prognosis in these patients. During the past fourteen years a number of mouse monoclonal antibodies have been raised against epitopes on the external domain of the human EGFR and these have been used not only to investigate growth factor-receptor interaction and the mechanism(s) of activation of the EGF receptor system but also for diagnostic and therapeutic applications in cancer. Several of the mouse antibodies have undergone clinical evaluation in Phase I and Phase II studies in patients with head and neck, lung or brain cancers including mAb EGFR1 (52,53), mAbs 225 and 528 (42,54), mAb 425 (EMD 55900 E Merk, 55-59) and mAb RG83852 (60). The aim of these studies, like the one presented here, was to determine whether treatment of cancer patients with anti-EGFR mAbs produced life threatening toxicities by binding to the EGFR expressed by normal tissues including liver and skin. The results of these studies have shown that mouse antibodies to the EGFR can be given safely to patients without toxicity. For example, Divigi and colleagues have treated patients with advanced squamous cell carcinoma of the lung with single doses of up to 300mg of mAb 225, including 4mg of indium 111-labeled 225, without any sign of toxicity (42). Furthermore, with doses of 40mg or greater they were able to image presumed sites of

metastasis greater or equal to 1cm in diameter (42).

We selected mAb ICR62 for a phase I clinical study since it was the most effective of a large number of rat mAbs we had made against the human EGFR, at inducing the regression of xenografts of head and neck, vulva and breast carcinomas grown in athymic mice (39,50,51). The results of this Phase I clinical study has indicated that mAb ICR62 can be given safely, at doses up to 100mg, to cancer patients without any life threatening toxicities. This finding is in agreement with the results of studies with mouse antibodies to the EGFR described above. Furthermore, in patients that had been given doses of 40 or 100mg of ICR62, significant amounts of therapeutic mAb were detected in the blood of these patients at 4 and 24 hours post ICR62 treatment. Moreover, in these patients, good localization of ICR62 was observed in metastatic lesions at 24 hours following treatment and penetration of antibody was greater in the tumours of patients treated with 100mg of the mAb. Importantly, the most accessible cells, i.e. those nearest the blood supply, are likely to be the most actively dividing cells and these were the most strongly stained. It is the proliferation of these cells which must be arrested. The maintenance of receptor blockade may require repeated treatment with antibody. Certainly, our experience using the xenograft model (39,50,51) points to the need to maintain sufficiently high blood levels for long enough to a) block EGFR function, b) recruit to the tumour and activate host effector cells and c) induce terminal differentiation. The data on the blood half-life of ICR62 obtained in this investigation suggest that twice weekly doses of 100mg may be sufficient to maintain a high enough level of this antibody for therapeutic activity. In the present investigation we were able to biopsy metastatic sites at only a single timepoint so we have no information concerning the stability of the antibody at the tumour cell surface or of the effects of treatment on the recruitment and activation of host immune effector cells.

When sera were tested for antibodies to rat immunoglobulin, we found that only four out of the twenty patients had responded following a single dose of mAb ICR62. Of these, only two were directed against the idiotype of ICR62. These results suggest that mAb ICR62 was not as immunogenic as the mouse antibodies to the EGFR that have been used previously in clinical studies. For example, Divigi and colleagues found all 19 lung cancer patients treated with a single dose of 1 to 300mg of mAb 225 developed human anti-mouse antibodies (42). Stasiaki and colleagues have also found that a single infusion or multiple infusions at monthly intervals of mAb EMD 55900 in glioma patients elicited human anti-mouse antibodies (57). On the other hand, these authors reported that following multiple infusions of glioma patients with mAb EMD 55900 at shorter intervals (three times/week, during 4 weeks or longer), human anti-mouse antibodies were not detectable in the

serum of these patients (57). The immunogenicity of anti-EGFR mAbs may however, be reduced by either production of chimeric or humanized version of the antibodies or by using different antibodies to the EGFR for the second and third treatment.

In summary, the results of this Phase I dose escalation study has indicated that the rat mAb ICR62, which acts as an EGF and TGF antagonist, a) can be administered safely in patients with squamous cell carcinoma, b) localizes efficiently to metastatic sites in patients with squamous cell carcinoma, and c) may therefore be useful in the treatment of a significant number of cancer patients whose tumours overexpress the EGF receptor. From the results of our preclinical studies in athymic mice, we have noted that to achieve tumour destruction repeated administration of EGFR mAbs may be required. For this reason, a phase II clinical study is planned using multiple doses of mAb ICR62.

TABLE 1

PATIENT No	M/F	L/HN STAGE	AGE AT PRESENTATION	PS WT	DATE OF DIAGNOSIS	PREVIOUS TREATMENT	DATE OF TREATMENT	DOSE	CTC TOXICITY (GRADE)	RESPONSE	DISEASE-FREE PROGRESSION PERIOD	DATE OF DEATH	HARA
1	M	HN	58	1 46	6/89	S 1990 R 1989 C 1989/92	28.10.92	2.5 mg	-	PR	1.5 months	11.2.93	-
2	F	HN	74	2 66	11/91	S 1992 R 1991	6.11.92	2.5mg	fever (0)	NC	1 month	1.12.92	-
3	F	HN	51	1 39	10/83	R 1985 C 1992	13.12.92	2.5mg	fever (0) BP (0)	PD	-	28.3.93	-
4	M	HN	49	1 60	7/90	S 1991 I 1991 C 1991/92	12.1.93	10 mg	ALT (0) fever (0) BP (0) rigor (1)	PD	-	13.3.93	-
5	M	HN	57	1 58	11/91	S 1991/92 R 1992 C 1992	9.2.93	10mg	rigor (0) fever (0)	PD	-	19.7.93	-
6	F	HN	46	2 43	12.3.92	S 1992 R 1992 C 1992	19.2.93	10mg	N&V (0) BP (0)	NC	-	16.3.93	-
7	M	L	66	1 53	27.11.92	-	1.3.93	20mg	rigor (0) fever (0) chest pain (0)	PD	-	21.8.93	-
8	M	L	61	1 71	13.8.84	lobectomy L/84 local RT 12/89 local RT 5/93 IM216 1/93	20.4.93	20mg	fever (0)	PD	-	2.6.93	-
9	F	L	56	1 61	15.4.88	Rad RT 1988 MVP 3/91-6/91 Local RT 4/93	7.6.93	20mg	cramps (0) fever ALT (0)	PD	-	14.9.93 +	-
10	M	HN	73	2 50	7/89	R 1989 C 1992	21.7.93	40mg	rigor (0) fever (0)	PD	-	12.12.93	-
11	F	HN	43	1 65	7.12.92	S 1993 R 1993 I 1993 C 1993	28.7.93	40mg	ALT (0) fever (0)	NC	-	LOST TO FOLLOW UP	-
12	M	L	68	1 43	13.6.91	Lobectomy 1991	17.8.93	40mg	fever (0) rigor (0) BP (0)	NC (PD 28.11.94)	1.5 months	30.11.94	-
13	M	L	70	1 84	19.6.91	Rad RT 5/91 Immunotherapy 91	21.8.93	100mg	BP (0) fever (0) rigor (0)	NC (PD 15.8.94)	12 months	Alive	-
14	M	HN	79	1 46	4.3.91	R 1991 I 1993 C 1993	2.9.93	100mg	rigor (0) fever (0) BP (0)	PD	-	11.12.93	-
15	M	L	65	1 53	21.2.93	CE 3/93-4/93 Rad RT 7/93	14.9.93	100mg	rigor (0) fever (0)	PD	-	24.12.93	-
16	M	HN	37	1 79	17.1.92	S 1992 R 1992 C 1992	23.9.93	100mg	fever (0) rigor (0)	PD	-	21.10.93 +	-
17	M	L	67	1 63	20.12.93	-	6.1.94	100mg	rigor (0) nausea (0) fever (0)	NC (PD 16.5.94)	4 months	Alive	-
18	F	HN	44	1 75	12.7.93	S 1990 R 1990 C 1993	11.1.94	100mg	rigor (0) fever (0)	NC	7 months	Alive	-
19	F	L	54	0 61	19.1.94	(also breast T)	3.2.94	100mg	rigor (0) N&V (0)	PD	-	11.4.94 +	-
20	M	L	71	1 96	13.1.94	-	21.7.94	100mg	-	NC (PD 19.9.94)	3 months	Alive	-

SUBSTITUTE SHEET (RULE 26)

References

1. Modjtahedi, H. and Dean C. The receptor for EGF and its ligands: Expression, prognostic value and target for therapy in cancer (Review). *Int. J. Oncol.*, 4, 1994, (in press).
2. Ullrich, A. and Schlessinger, J. *Cell*. 61, 203-212 (1990).
3. Spaargaren, M., Defize, L.H.K., Boonstra, J. and de Laat, S.W. *J. Biol. Chem.*, 266, 1733-1739 (1991).
4. Prigent, S.A. and Lemoine, N.R. The type 1 EGFR-related family of growth factor receptors and their ligands. *Progress in Growth Factor Research*, 4: 1-24 1992.
5. Aboud-Pirak, E., Hurwitz, E., Pirak, M.E., Bellot, F., Schlessinger, J. and Sela, M. *J. Natl. Cancer. Inst.*, 80, 1605-1611 (1988).
6. Kamata, N., Kazuhiro, C., Rikimaru, K., Horikoshi, M., Enomoto, S. and Kuroki, T. *Cancer Res.*, 46, 1648-1653 (1986).
7. Sato, J.D., Kawamoto, T., Le, A.D., Mendelsohn, J. Polikoff, J. and Sato, J.H. *Mol. Biol., Med.* 1, 511-514 (1983).
8. Fan, Z., Masui, H., Altas, I. and Mendelsohn, J. *Cancer Res.* 53, 4322-4328 (1993).
9. Cowley, G., Smith, J.A., Gusterson, B., Hendler, F., and Ozanne, B. The amount of EGF receptor is elevated on squamous cell carcinomas. *Cancer Cells*, 1: 5-10, 1984.
10. Hendler, F.J. and Ozanne B.W. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. Clin. Invest.*, 74: 647-651, 1984.
11. Ozanne, B., Richards, C.S., Hendler, F., Burns, D. and Gusterson, B. Overexpression of the EGF receptor is a hallmark of squamous cell carcinomas. *J. Pathol.*, 149: 9-14, 1986.
12. Gullick, W.J. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *British Medical Bulletin*, Vol 47: No.1 87-98 1991.
13. Harris, A.L., Nicholson, S., Sainsbury, R., Wright, C and Farndon, J.R. Epidermal growth factor receptor and other oncogenes as prognostic markers. *J. Natl. Cancer Inst. Monogr.* 11: 181-87, 1992.
14. Sporn, M.B. and Roberts, A.B. Autocrine growth factors and cancers. *Nature*, 313: 745-747, 1985.
15. Di Marco, E., Pierce, J. H., Fleming, T. P., Kraus, M. H., Molloy, C. J., Aaronson, S. A. and DiFore, P. P. Autocrine interaction between TGF α and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. *Oncogene*, 4: 831-838, 1989.
16. Yoshida, K., Kyo, E., Tsuda, T., Tsujino, T., Ito, M. and Tahara, E. EGF and TGF α , the ligands of hyperproduced EGFR in human esophageal carcinoma cells act as a autocrine growth factor. *Int. J. Cancer* 45: 131-135, 1990.
17. Morishigie, K.I., Kurachi, H., Amemiya, K., Fujita, Y., Yamamoto, T., Miyake, A. and Tanizawa, O. Evidence for the involvement of TGF α and EGFR autocrine mechanism

- in primary human ovarian cancers in vitro. Cancer Res., 51: 5322-5328, 1991.
18. Masui, H., Kawamoto, T. Sato, J.D., Wolf, B., Sato, G. and Mendelsohn, J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Research. 44: 1002-1007, 1984.
19. Van de Vijvert, M., Kumar, R. and Mendelsohn, J. Ligand-induced activation of A431 cell epidermal growth factor receptors occurs primarily by an autocrine pathway that acts upon receptors on the surface rather than intracellularly. J. Biol. Chem., 266: 7503-7508, 1991.
20. Rodeck, U., Herlyn, M., Herlyn, D., Molthoff, C., Atkinson, B., Varello, M., Steplewski, Z. and Koprowski, H. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: Immunological mediated and effector cell-independent effects. Cancer Research. 47: 3692-3696, 1987.
21. Imanishi, K., Yamaguchi, K., Kuranami, M., Kyo, E., Hozumi, T. and Abe, K. Inhibition of growth of human lung adenocarcinoma cell lines by anti-transforming growth factor- α monoclonal antibody. J. Natl. Cancer Inst., 81: 220-223, 1989.
22. Fendly, B.M., Winget, M., Hudziak, R.M., Lipari, M.T., Napier, M.A. and Ullrich, A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res., 50: 1550-1558, 1990.
23. Sato, H. and Sato, J.D. Growth factor receptor monoclonal antibodies and cancer immunotherapy. J. Nat. Cancer Inst. 81: No21 1600-1601, 1989.
24. Mendelsohn, J. Epidermal growth factor receptor as a target for therapy with antireceptor monoclonal antibodies. J. Natl. Cancer Inst. Monogr. 13: 125-131, 1992.
25. Modjtahedi, H., Styles, J., Box, G., Eccles, S., Gusterson, B., and Dean, C. Antitumour activity of rat Mabs to the human receptor for EGF. In, Mutant Oncogenes: Targets for Therapy? Eds. Epenetos, A.A. and Lemonie, N.R. Chapman & Hall, London pp35-47, 1993.
26. Modjtahedi, H., Styles, J.M., and Dean, C.J. The human EFG receptor as a target for cancer therapy : six new rat mAbs against the receptor on the breast carcinoma MDA-MB 468. Br. J. Cancer, 67: 247-253, 1993.
27. Dean, C.J., Styles, J.M., Gyure, L.A., Peppard, J., Hobbs, S.M., Jackson, E., and Hall, J.G. The production of hybridomas from the gut associated lymphoid tissue of tumour bearing rats. I. Mesenteric nodes as a source of IgG producing cells. Clin.exp.Immunol., 57: 358-364, 1984.
28. Hudson, D. L., Weiland, K. L., Dooley, T. P., Simon, M. and Watt, F. M. Characterisation of eight monoclonal antibodies to involucrin. Hybridoma, 11: 367-379, 1992.
29. Modjtahedi, H., Eccles, S. Box, G, Styles, J. and Dean

- C.J. Antitumour activity of combinations of antibodies directed against different epitopes on the extracellular domain of the human EGF receptor. *Cell BioPhysics* 21/22: 1993 (in press).
- 5 30. Ormerod, M.G. Analysis of DNA. In, *Flow Cytometry*, Ed M. G. Ormerod Oxford University Press, New York, 69-87, 1990.
- 10 31. Banks-Schlegel, S. and Green, H. Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. *J. Cell Biol.*, 90: 732-737, 1981.
- 15 32. Watt, F. M. and Green, H. Involucrin synthesis is correlated with cell size in human epidermal cultures. *J. Cell Biol.*, 90: 738-742, 1981.
- 20 33. Walts, A. E. and Said, J. W. Involucrin, a marker of squamous and urothelial differentiation. An immunohistochemical study on its distribution in normal and neoplastic tissues. *J. Path.*, 145: 329-340, 1985.
- 25 34. Moll, R., Franke, W. W., Schiller, D. L., Geiger, B. and Krepler, R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell*, 31: 11-24, 1982.
- 30 35. Lane, E. B. and Alexander, C. M. Use of keratin antibodies in tumor diagnosis. *Cancer Biology*, 1: 165-179, 1990.
- 35 36. Léger, O.J.P. and Dean, C.J. *Mol. Immunol.* 28, 673-680, (1991).
- 40 37. Tachikawa, T., Boman, B., Mendelsohn, J., and Masui, H., Tumour cytotoxicity by anti-epidermal growth factor receptor EGFR monoclonal antibody mAb. *Proc.Amer.Assoc. Cancer Res.* 33:345, 1992.
- 45 38. Bacus, S.S., Stancovski, H., Huberman, E., Chin, D., Hurwitz, E., Mills, G.B., Ullrich, A., Sela, M. and Yarden, Y. Tumor-inhibitory monoclonal antibodies to the HER-2/neu receptor induce differentiation of human breast cancer cells, 52: 2580-2589, 1992.
- 50 39. Modjtahedi, H., Eccles, S.A., Box, G., Style, J., and Dean C., Immunotherapy of human human tumor xenografts overexpressing the EGF receptor with rat antibodies that block growth factor receptor interaction. *Br.J.Cancer* 67: 254-261, 1993.
- 55 40. Bruggemann, M., Teale, C., Clark M., Bindon C., and Waldmann H., A matched set of rat/mouse chimeric antibodies. *J.Immunol.* 142:3145-3150, 1989.
41. Mendelsohn, J., The epidermal growth factor receptor as a target for therapy with antireceptor monoclonal antibodies. *Seminars in Cancer Biology* 1:339-344, 1990.
42. Divigi, C.R., Welt, S., Kris, M., Real, F.X., Yeh, S.D.J., Gralla, R., Merchant, B., Schweighart, S., Unger, M., Larson, S.M., and Mendelsohn, J. Phase I and Imaging Trial of Indium 111-Labeled Anti-Epidermal Growth Factor Receptor Monoclonal Antibody 225 in patients with Squamous Cell Lung Carcinoma. *J. Natl. Cancer Inst.* 83:97-104, 1991.

43. Pierce, G.B., and Speers, W.C., Tumours as Caricatures of the Process of Tissue Renewal: Prospects for Therapy by Directing Differentiation. *Cancer Res.* 48:1996-2004, 1988.
- 5 44. Schreiber, A.B., Libermann, T.A., Lax, I., Yarden, Y. and Schlessinger, J. *J. Biol. Chem.* 258, 846-853 (1983).
45. Fernandez-Pol, J.A. *J. Biol. Chem.*, 260, No 8, 5003-5011 (1985).
- 10 46. Koland, J.G and Cerione, R.A. *J. Biol. Chem.*, 263, 2230-2237 (1988).
47. Carraway, K.L., and Cerione R.A. *J. Biol. Chem.*, 268, 23860-23867 (1993).
48. Yarden, Y., and Schlessinger, J. *Biochemistry* 26, 1434-1442 (1987).
- 15 49. Weber, W., Bertics, P.J., and Gill G.N. *J. Biol. Chem.*, 259, 14631-14636 (1984).
50. Dean CJ, Modjtahedi H, Eccles S, et al: Immunotherapy with antibodies to the EGF receptor. *Int J Cancer* 8:103-107 1994.
- 20 51. Modjtahedi H, Eccles S, Sandle J, et al: Differentiation or immune destruction: two pathways for therapy of squamous cell carcinomas with antibodies to the epidermal growth factor receptor. *Cancer Research.* 54:1695-1701, 1994.
- 25 52. Soo KC, Ward M, Roberts KR, et al., Radioimmunoscinigraphy of squamous carcinomas of the head and neck. *Head and Neck Surgery* 9:349-352, 1987.
53. Kalofonos HP, Pawlikowska TR, Hemingway A, et al: Antibody guided diagnosis and therapy of brain gliomas using radiolabeled monoclonal antibodies against epidermal growth factor receptor and placental alkaline phosphatase. *J Nucl Med*, 30:1636-1645, 1989.
- 30 54. Baselga J, Scott A, Pfister D, et al: Comparative pharmacology in Phase I and imaging trials utilizing anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (MAbs) labeled with ¹³¹I or ¹¹¹IN. *Proc Annu Meet Am Soc Clin Oncol*, 12:A368, 1993.
- 35 55. Brady LW, Miyamoto C, Woo DV, et al: Malignant astrocytomas treated with iodine-125 labelled monoclonal antibody 425 against epidermal growth factor receptor: a phase II trial. *Int J Radiation Oncology Biol Phys*, 22:225-230, 1991.
- 40 56. Magdelenat H, Delattre JY, Mady E, et al: A phase I study of the anti-EGFR monoclonal antibody 425 in patients with malignant gliomas. *J Tumor Marker Oncol* 6(4):60, 1991.
- 45 57. Stasiecki P, Kemshead JT, Westphal M, et al: The development of HAMA in glioma patients can be suppressed by administration of murine MAb in short time intervals. *Proc Annu Meet Am Assoc Cancer Res*, 34:A2835, 1993.
- 50 58. Bilzer T, Stasiecki P, Vega F, et al: Immunotherapy of malignant gliomas with the anti-EGFR monoclonal antibody. *Proc Annu Meet Am Assoc Cancer Res*, 34:A2877, 1993.
- 55

59. Dadparvar S, Krishna L, Miyamoto C, Brady LW, Brown SJ, Bender H, Slizofski WJ, Eshleman J, Chevres A, Woo DV: Indium-111-labeled anti-EGFr-425 scintigraphy in the detection of malignant gliomas. Cancer Suppl 73:884-889, 1994.
- 5 60. Shin DM, Perez-Soler R, Donato NJ, et al: Tumor epidermal growth factor receptor (EGFR) saturation and proliferating cell nuclear antigen (PCNA) expression in patients treated with anti-EGFR monoclonal antibody (MoAb) RG83852 (in Phase I clinical study). Proc Annu Meet Am Soc Clin Oncol, 12:A940, 1993.
- 10

Sequence Listings ICR62

ICR62VH

30	60
ATG GGA TGG ATC TGT ATC ATC TTT CTT GTG GCA ACA GCT ACA GGT GGC CAC TCC CAG GTC	
Met Gly Trp Ile Cys Ile Ile Phe Leu Val Ala Thr Ala Thr Gly Gly His Ser Gln Val	
90	120
AAC CTA CTG CAG TCT GGG GCT GCA CTG GTG AAG CCT GGG GCC TCT GTG AAG TTG TCT TGC	
Asn Leu Leu Gln Ser Gly Ala Ala Leu Val Lys Pro Gly Ala Ser Val Lys Leu Ser Cys	
150	180
AAA GGT TCT GGT TTT ACA TTC ACT [GAC TAC AAG ATA CAC] TGG GTG AAG CAG AGT CAT GGA	
Lys Gly Ser Gly Phe Thr Phe Thr [Asp Tyr Lys Ile His] Trp Val Lys Gln Ser His Gly	
210	240
AAG AGC CTT GAG TGG ATT GGG [TAT TTT AAT CCT AAC AGT GGT TAT AGT ACC TAC AAT GAA	
Lys Ser Leu Glu Trp Ile Gly [Tyr Phe Asn Pro Asn Ser Gly Tyr Ser Thr Tyr Asn Glu	
270	300
AAG TTC AAG AGC [AAG GCC ACA TTG ACT GCA GAC AAA TCC ACC GAT ACA GCC TAT ATG GAG	
Lys Phe Lys Ser [Lys Ala Thr Leu Thr Ala Asp Lys Ser Thr Asp Thr Ala Tyr Met Glu	
330	360
CTT ACC AGT CTG ACA TCT GAG GAC TCT GCA ACC TAT TAC TGT ACA AGA [CTA TCC CCA GGG	
Leu Thr Ser Leu Thr Ser Glu Asp Ser Ala Thr Tyr Tyr Cys Thr Arg [Leu Ser Pro Gly	
390	420
GGT TAC TAT GTT ATG GAT GCC [TGG GGT CAA GGA GCT TCA GTC ACT GTC TCC TCA GCC CAA	
Gly Tyr Tyr Val Met Asp Ala [Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser Ala Gln	
450	
ACA ACA GCC CCA TCT GTC TAT CCA CTG GCT CCT GGA	
Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Gly	

SUBSTITUTE SHEET (RULE 26)

ICR62VK

30	60
ATG ATG GCT CCA GTC CAG CTC TTA GGG CTG CTG CTG ATT TGG CTC CCA GCC ATG AGA TGT	
Met Met Ala Pro Val Gln Leu Leu Gly Leu Leu Leu Ile Trp Leu Pro Ala Met Arg Cys	
90	120
GAC ATC CAG ATG ACC CAG TCT CCT TCA TTC CTG TCT GCA TCT GTG GGA GAC AGA GTC ACT	
Asp Ile Gln Met Thr Gln Ser Pro Ser Phe Leu Ser Ala Ser Val Gly Asp Arg Val Thr	
150	180
ATC AAC TGC [AAA GCA AGT CAG AAT ATT AAC AAT TAC TTA AAC] TGG TAT CAG CAA AAG CTT	
Ile Asn Cys [Lys Ala Ser Gln Asn Ile Asn Asn Tyr Leu Asn] Trp Tyr Gln Gln Lys Leu	
210	240
GGA GAA GCT CCC AAA CGC CTG ATA TAT [AAT ACA AAC AAT TTG CAA ACA] GGC ATC CCA TCA	
Gly Glu Ala Pro Lys Arg Leu Ile Tyr [Asn Thr Asn Asn Leu Gln Thr] Gly Ile Pro Ser	
270	300
AGG TTC AGT GGC AGT GGA TCT GGT ACA GAT TAC ACA CTC ACC ATC AGC AGC CTG CAG CCT	
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro	
330	360
GAA GAT TTT GCC ACA TAT TTC TGC [TTG CAG CAT AAT AGT TTT CCC ACG] TTT GGA GCT GGG	
Glu Asp Phe Ala Thr Tyr Phe Cys [Leu Gln His Asn Ser Phe Pro Thr] Phe Gly Ala Gly	
390	420
ACC AAG CTG GAA CTG AAA CGG GCT GAT GCT GCA CCA ACT GTA TCT ATC TTC CCA CCA TCC	
Thr Lys Leu Glu Leu Lys Arg Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser	
AAA TCG	
Lys Ser	

SUBSTITUTE SHEET (RULE 26)

Sequence Listings ICR64

ICR64VH

30	60
ATG AAG TTG TTG CTA AAC TGG GTT TTT CTA GTA ACA CTT TTA AAT GGT ATT CAG TGT GAG	
Met Lys Leu Leu Leu Asn Trp Val Phe Leu Val Thr Leu Leu Asn Gly Ile Gln Cys Glu	
90	120
GTG AAG CTG GTA GAA TAT GGA GGA GGT TTG GTG CAG CCT GGG CTT CTC TCA GAC CTC TCC	
Val Lys Leu Val Glu Tyr Gly Gly Gly Leu Val Gln Pro Gly Leu Leu Ser Asp Leu Ser	
150	180
TGC GAA GCT TCT GGA TTC ATG TTC AGT [GAT TTC TTC ATG GAG] TGG ATC CGA CAG GCT CCA	
Cys Glu Ala Ser Gly Phe Met Phe Ser [Asp Phe Phe Met Glu] Trp Ile Arg Gln Ala Pro	
210	240
GGG AAA GGA CTG GAG TGG ATT GCA [GCA AGT AGA AAC AAA GCT AAC GAT TAT TCA GCA GTG	
Gly Lys Gly Leu Glu Trp Ile Ala [Ala Ser Arg Asn Lys Ala Asn Asp Tyr Ser Ala Val	
270	300
TAC AGT GCA TCT GTG AAG GAC [CGA TTC ACC ATC TCA AGG GAT TCT CAC AAA AGC ATC CTC	
Tyr Ser Ala Ser Val Lys Asp [Arg Phe Thr Ile Ser Arg Asp Ser His Lys Ser Ile Leu	
330	360
TAT CTT CAG ATG AAC ACA CTC AAA CCT GAG GAT ACT GCC ATT TAT TAC TGT GCA AGA [GAT	
Tyr Leu Gln Met Asn Thr Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys Ala Arg [Asp	
390	420
TAT TAC CAA AGT GGC TTG TTT GAT TAC [TGG GGC CAA GGA GTC ATG GTC ACA GTC TCC TCA	
Tyr Tyr Gln Ser Gly Leu Phe Asp Tyr [Trp Gly Gln Gly Val Met Val Thr Val Ser Ser	

SUBSTITUTE SHEET (RULE 26)

35

ICR64VK

30 60
 ATG GGT GTG CCC ACT CAG CTC CTG GGG TTG TTG CTG CTG TGG ATA ACA GAT AGA ATA TGT
 Met Gly Val Pro Thr Gln Leu Leu Gly Leu Leu Leu Trp Ile Thr Asp Arg Ile Cys
 90 120
 GAC ATC CAG ATG ACA CAG TCT CCA GCT TCC CTG TCT GCA TCT CTG GGA GAA ACT GTC ACC
 Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Leu Gly Glu Thr Val Thr
 150 180
 ATC GAA TGT [CTA GTA AGT GAA GAC ATT TAC AGT AAT TTA GCG] TGG TAT CAG CAG AAG CCA
 Ile Glu Cys [Leu Val Ser Glu Asp Ile Tyr Ser Asn Leu Ala] Trp Tyr Gln Gln Lys Pro
 210 240
 GGG AAA TCT CCT CAG CTC CTG ATC TAT [GAT GCA AGT AGC TTG CAA GAT] GGG GTC CCA TCA
 Gly Lys Ser Pro Gln Leu Leu Ile Tyr [Asp Ala Ser Ser Leu Gln Asp] Gly Val Pro Ser
 270 300
 CGG TTC AGT GGC AGT GAA TCT GGC ACA CAG TAT TCT CTC GAG ATC AAC AGC CTG CAA TCT
 Arg Phe Ser Gly Ser Glu Ser Gly Thr Gln Tyr Ser Leu Glu Ile Asn Ser Leu Gln Ser
 330 360
 GAA GAT GCC GCG ACT TAT TTC TGT [CAA CAG CAT CAT GAT TAT CCT CGG ACG] TTC GGT GGA
 Glu Asp Ala Ala Thr Tyr Phe Cys [Gln Gln His His Asp Tyr Pro Arg Thr] Phe Gly Gly

 GGC ACC AAG CTG GAA TTG AAA
 Gly Thr Lys Leu Glu Leu Lys

SUBSTITUTE SHEET (RULE 26)

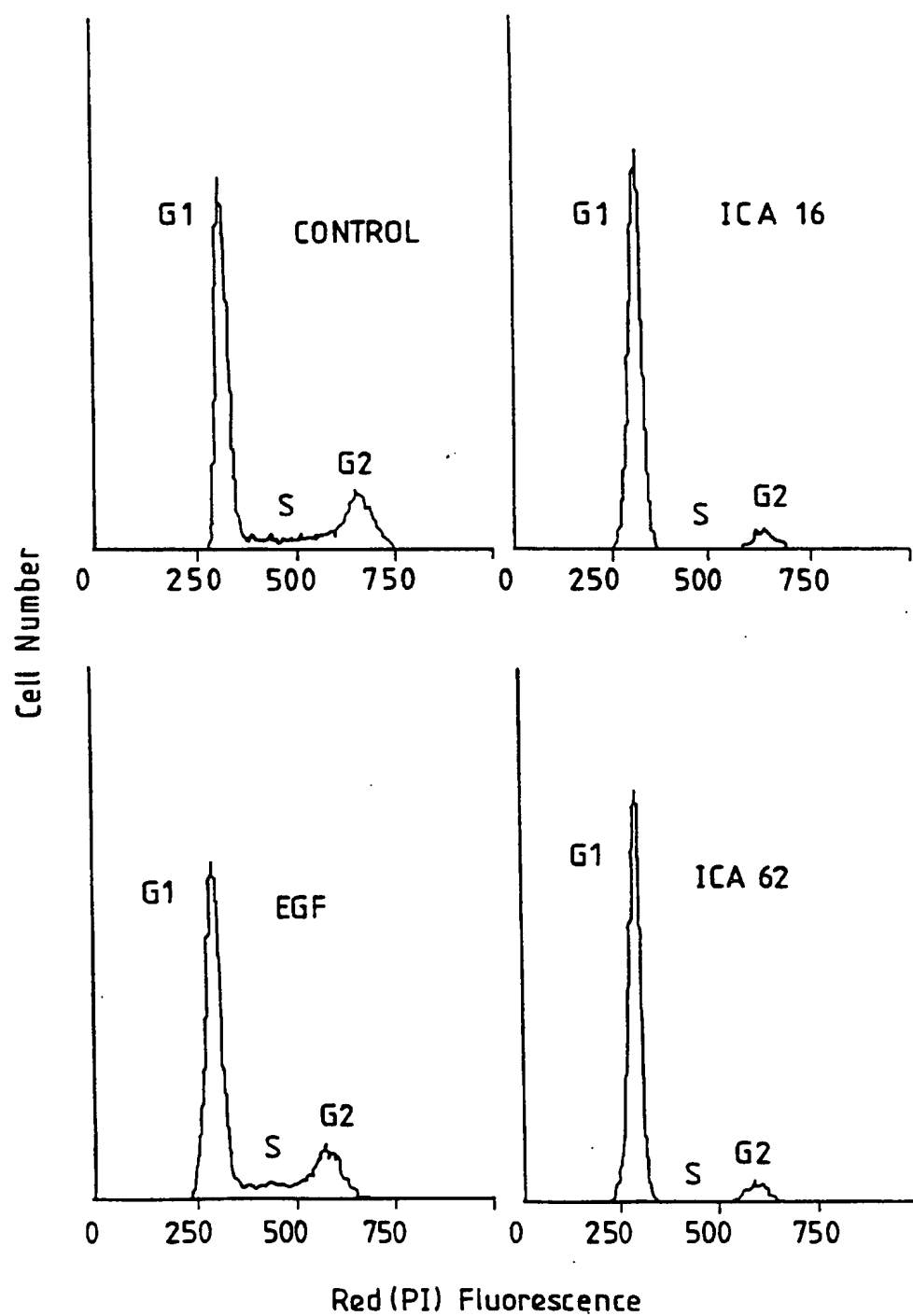
CLAIMS:

1. Antibodies to EGF receptor, or fragments thereof, for therapeutic use.
- 5 2. Antibodies according to claim 1 wherein the antibodies are antibodies ICR62, ICR64 or ICR16, or fragments thereof.
- 10 3. A mutant, derivative, or functional equivalent of the antibodies according to claim 1 or claim 2.
- 15 4. Antibodies according to claim 3 wherein the antibodies are humanised, having framework regions based on or derived from a human antibody and having complementary determining regions (CDRs) derived from a non-human antibody.
- 20 5. Antibodies according to claim 4 wherein the antibody has complementary determining regions (CDRs) derived from the ICR62VH, ICR62VK, ICR64VH or ICR64VK sequences shown in the accompanying sequence listing.
- 25 6. A fragment of an antibody to EGF receptor having the property of inducing terminal differentiation in tumour cells.
- 30 7. A fragment according to claim 6 wherein the fragment is a Fab fragment.
- 35 8. Use of the antibodies or fragments of antibodies as defined in any one of claims 1 to 7 in the preparation of a medicament for inducing terminal differentiation in tumour cells.
- 40 9. The use according to claim 8 wherein the cells are characterised by over-expressing EGF receptor.
- 45 10. The use according to claim 8 or claim 9 wherein the tumour cells are bladder, brain, head, neck, pancreas, lung, breast, or ovary tumour cells.
- 50 11. Isolated nucleic acid encoding an antibody or fragment of an antibody according to any one of claims 1 to 7.
- 55 12. An expression vector comprising the DNA of claim 11, the DNA being operably linked to control sequences for expressing the DNA.
13. Host cells transformed by the expression vector of claim 12.
14. A pharmaceutical composition comprising one or more of the antibodies or fragments of antibodies as defined in any one of claim 1 to 7.
15. A pharmaceutical composition according to claim 14 comprising from 1 to 300mg of the antibodies.

SUBSTITUTE SHEET (RULE 26)

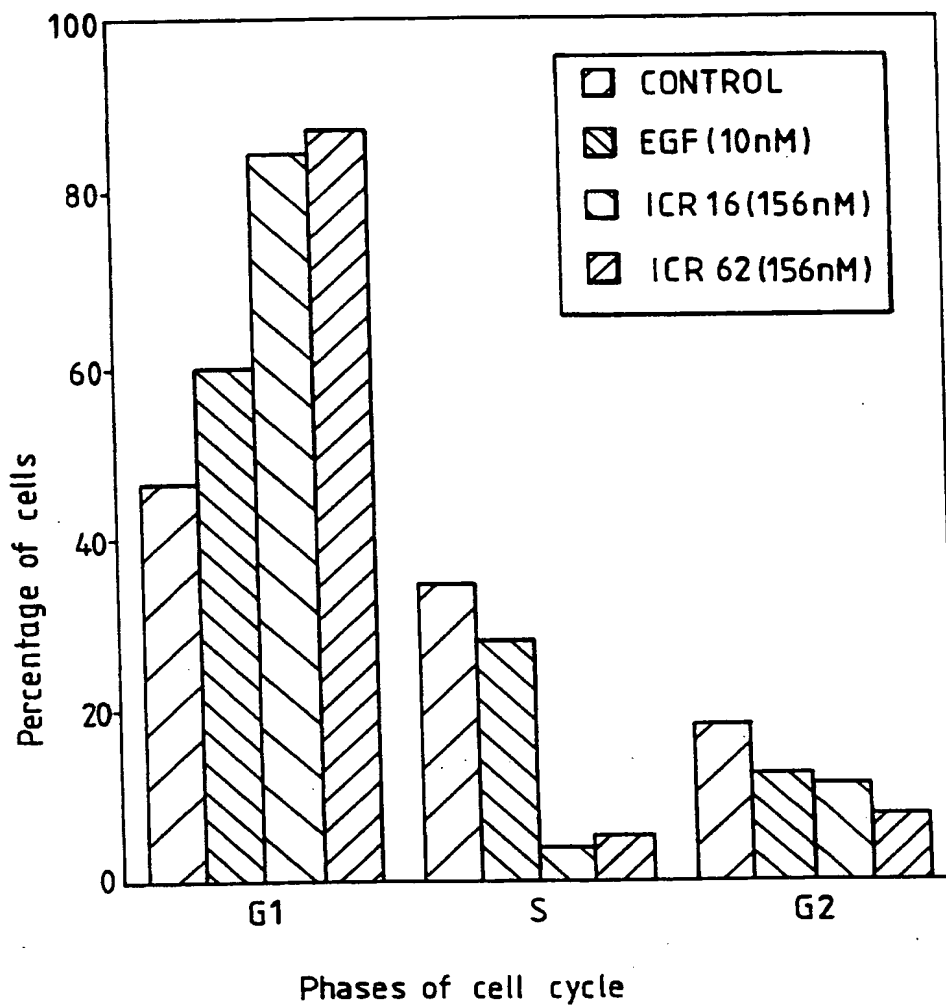
16. A pharmaceutical composition according to claim 14 or claim 15 wherein the antibodies or fragments of antibodies are conjugated to a cytotoxic compound, drug or toxin, or a label.
- 5 17. A functional mimetic of an antibody or fragment of an antibody as defined in any one of claims 1 to 7.
- 10 18. The use of an antibody or fragment of an antibody as defined in any one of claims 1 to 7 for the design or synthesis of peptide or non-peptide compounds which are functional mimetics of the antibody

1/12

*Fig.1.*

SUBSTITUTE SHEET (RULE 26)

2/12

*Fig.2.*

SUBSTITUTE SHEET (RULE 26)

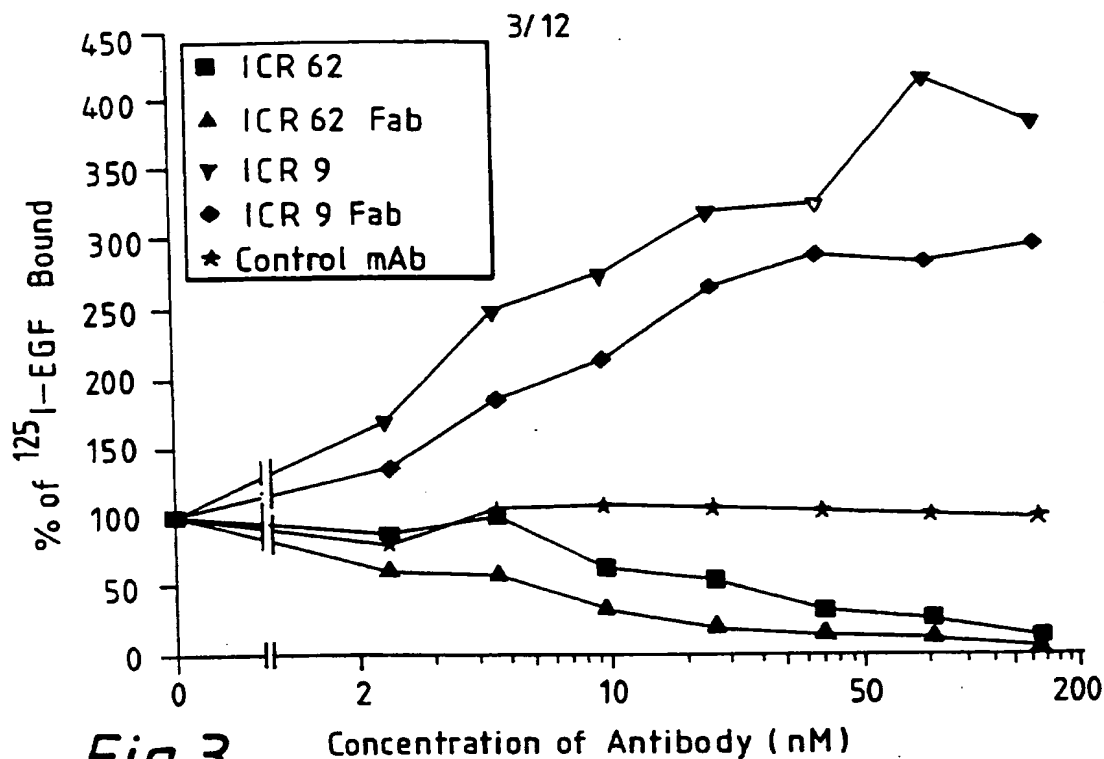


Fig.3.

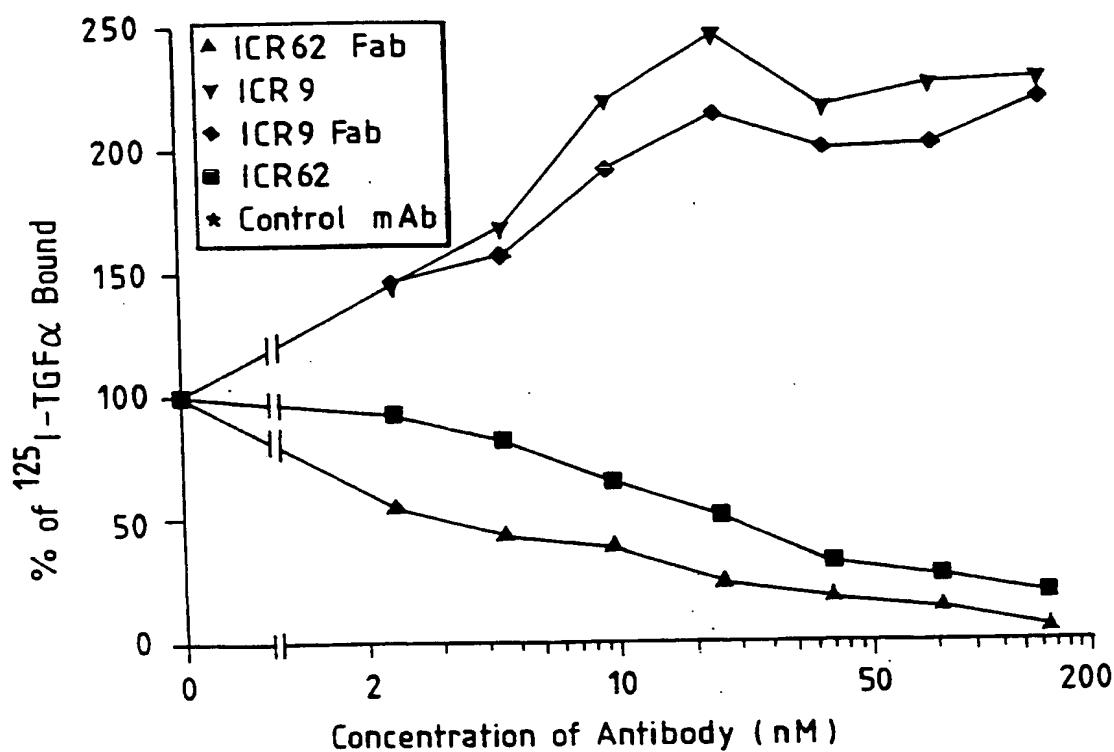


Fig.4.

SUBSTITUTE SHEET (RULE 26)

4/12

Schatchard analysis of 125 I-EGF binding to the EGF receptor on EJ cells
in the presence or absence of antibody ICR 9

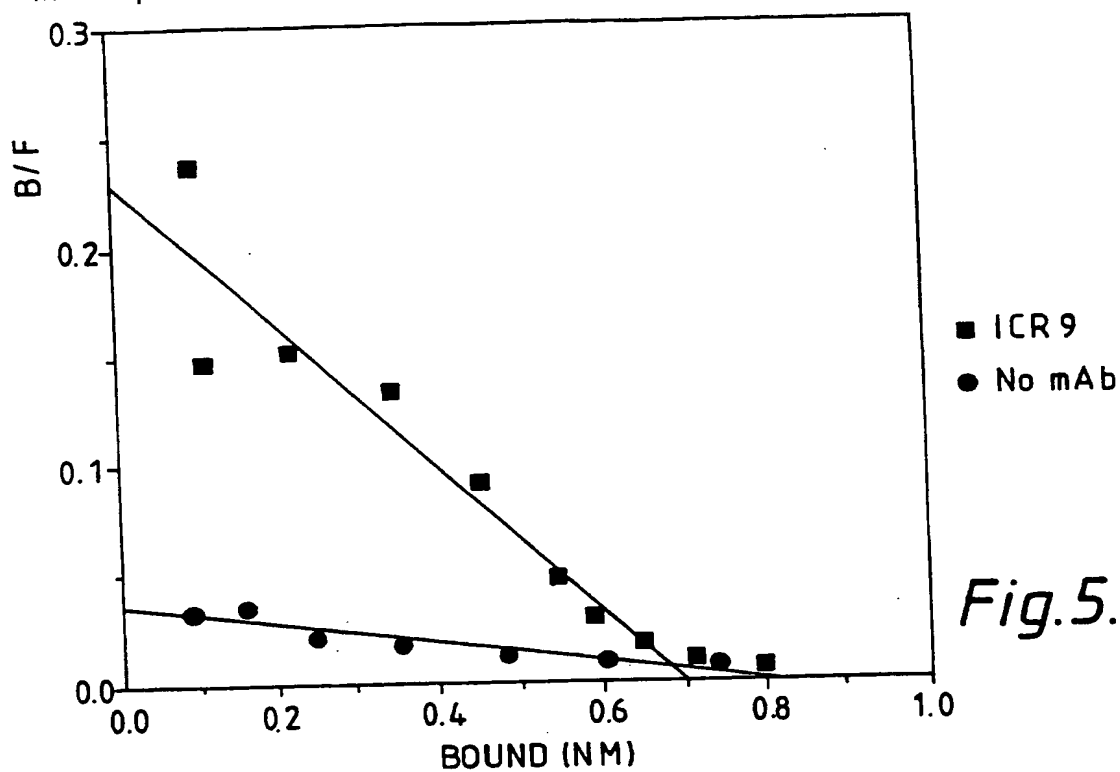


Fig.5.

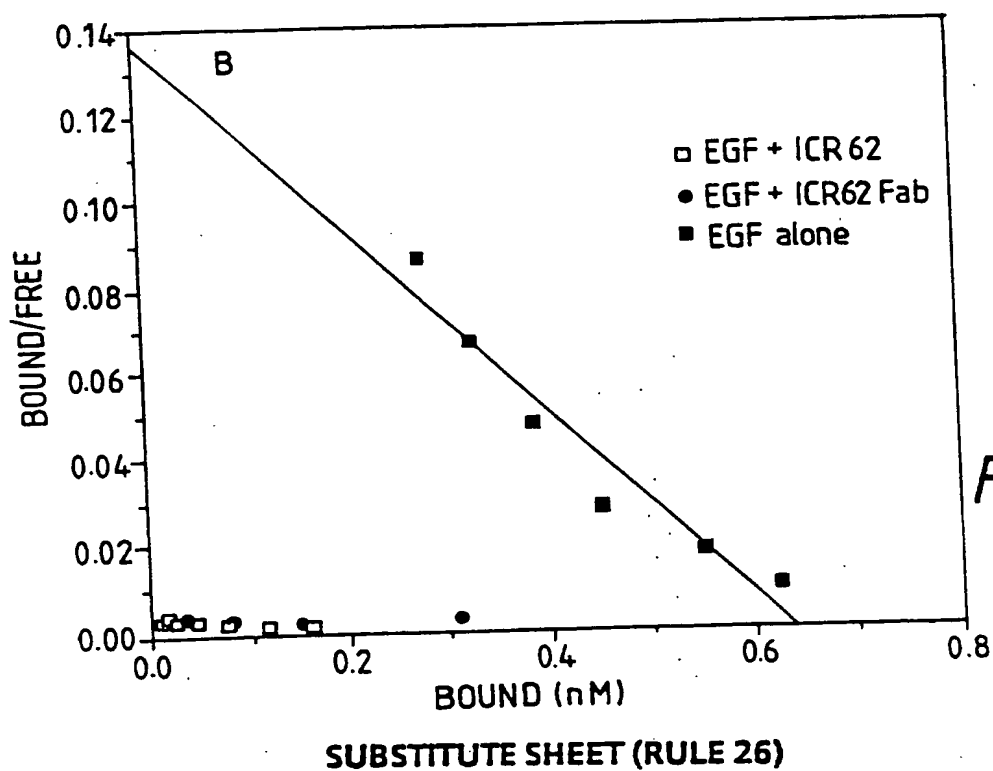
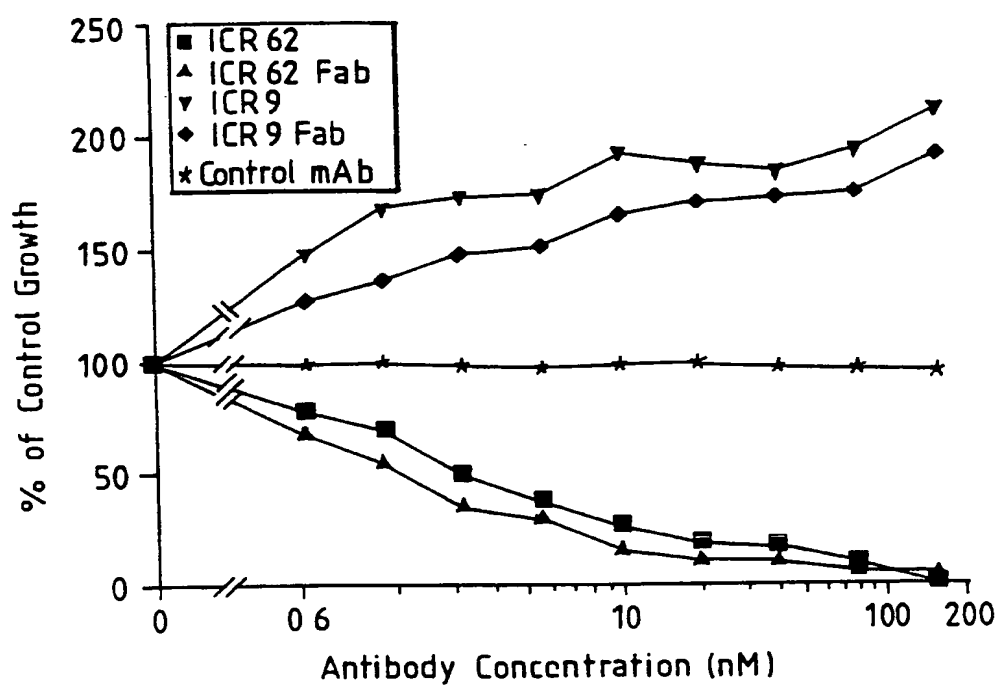


Fig.6.

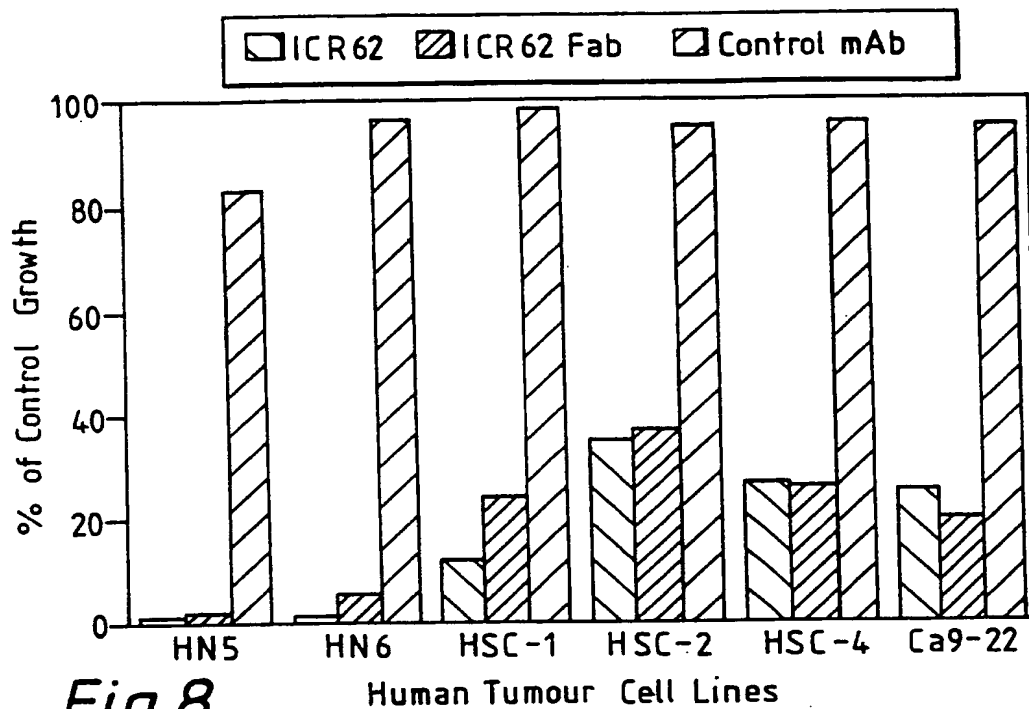
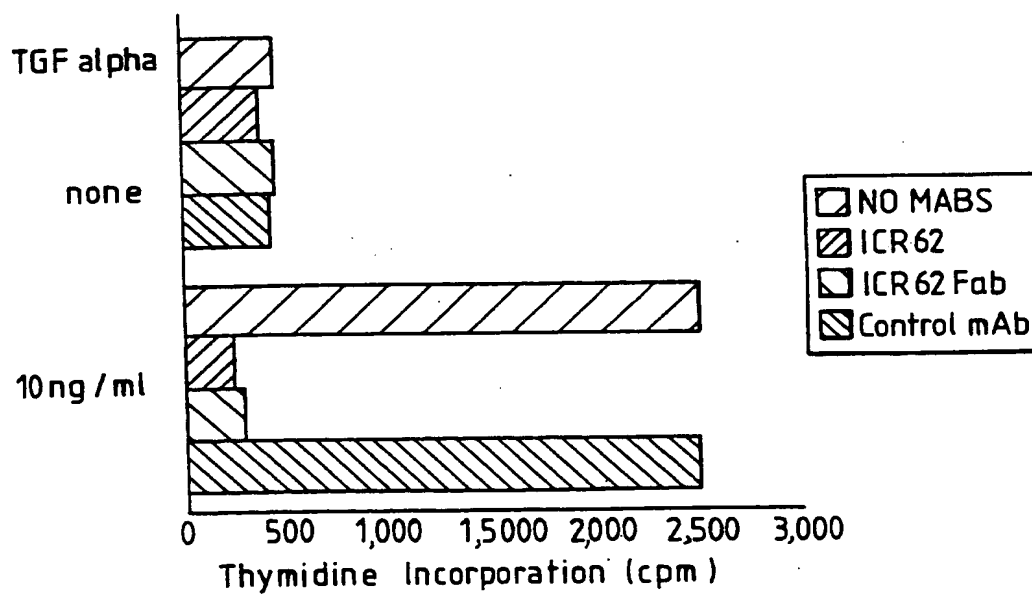
SUBSTITUTE SHEET (RULE 26)

5/12

*Fig.7.*

SUBSTITUTE SHEET (RULE 26)

6/12

*Fig.8.**Fig.9.*

SUBSTITUTE SHEET (RULE 26)

7/12

Fig.10A.

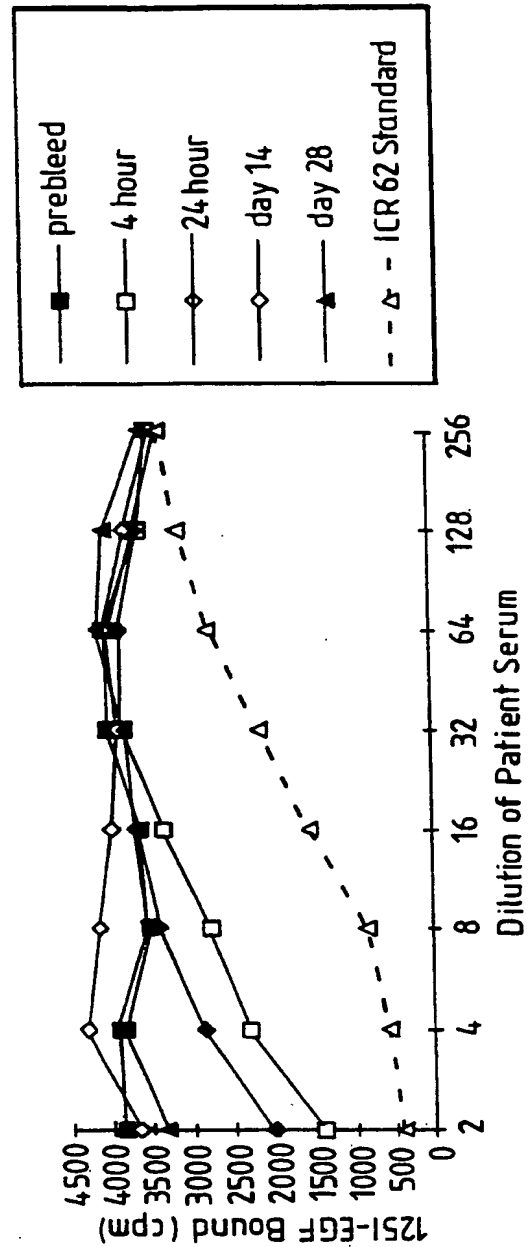
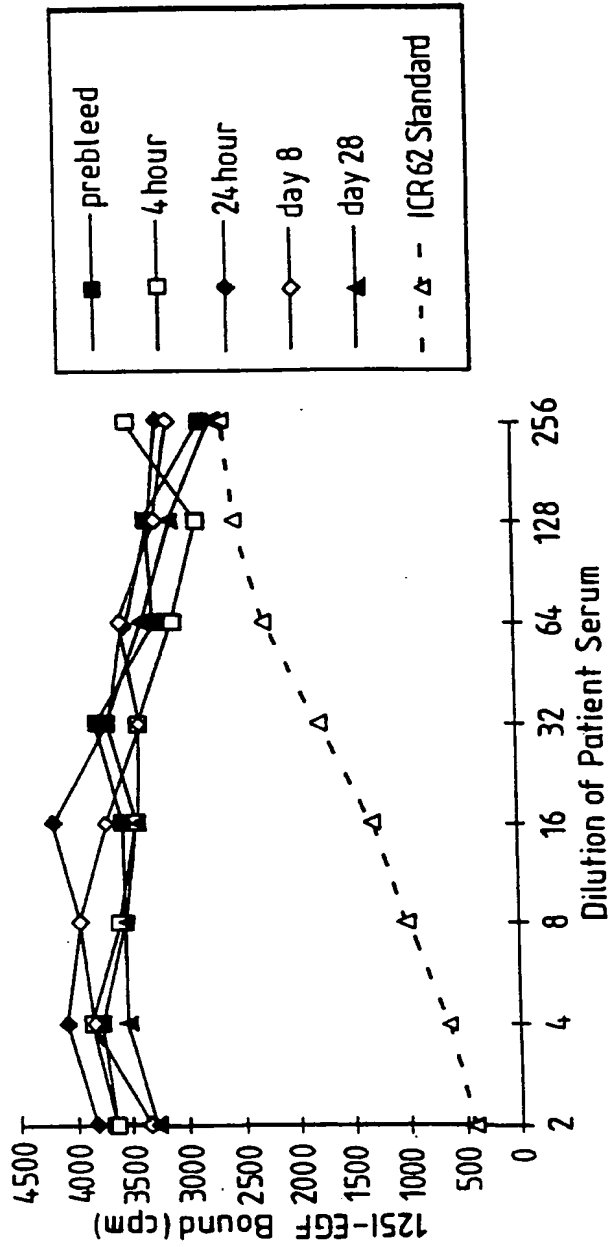


Fig.10B.

8 / 12

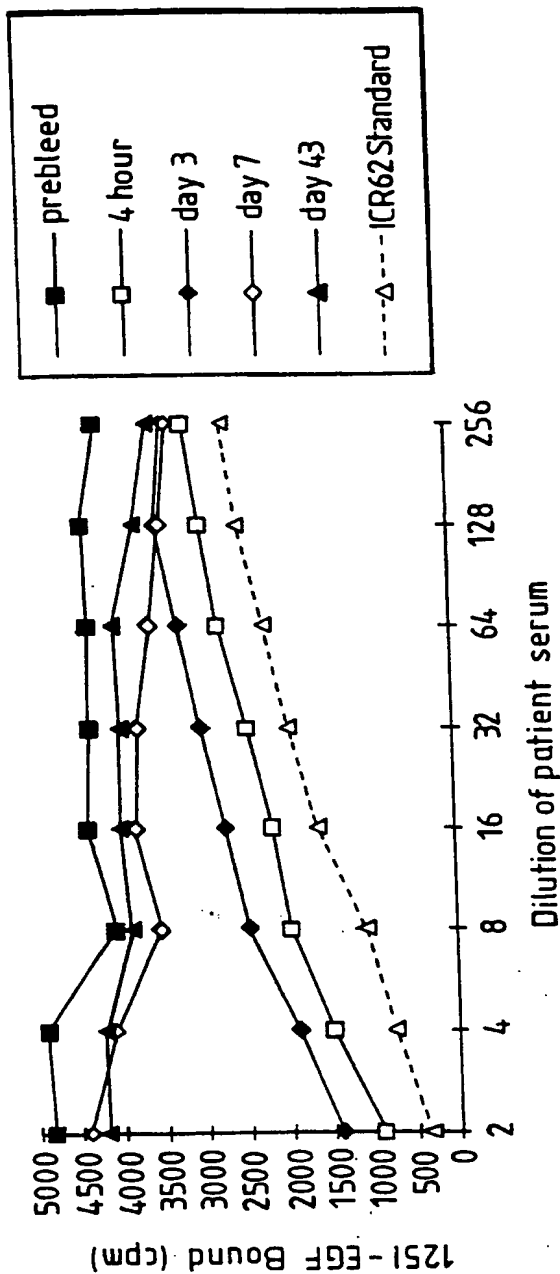


Fig.10c.

SUBSTITUTE SHEET (RULE 26)

9/12

Fig.11A.

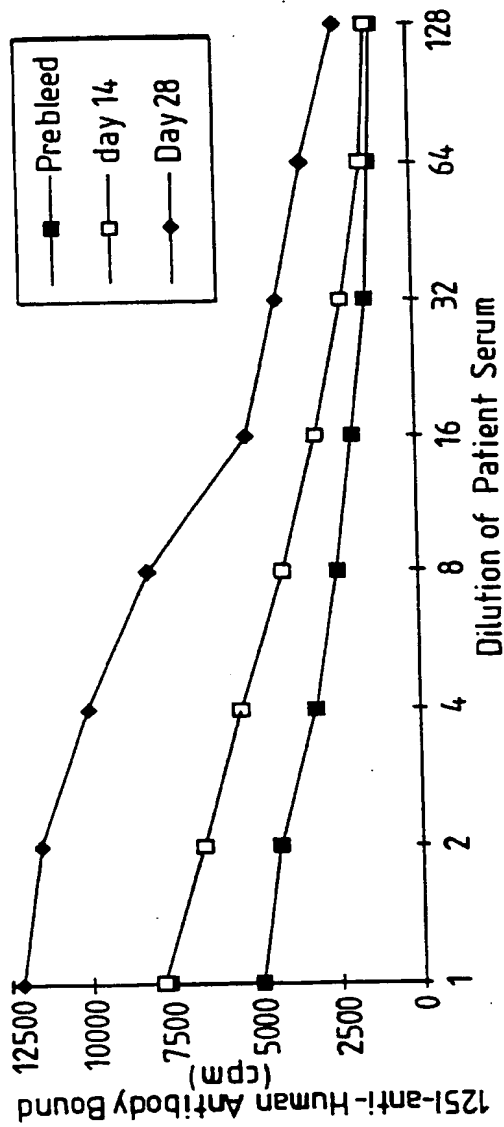
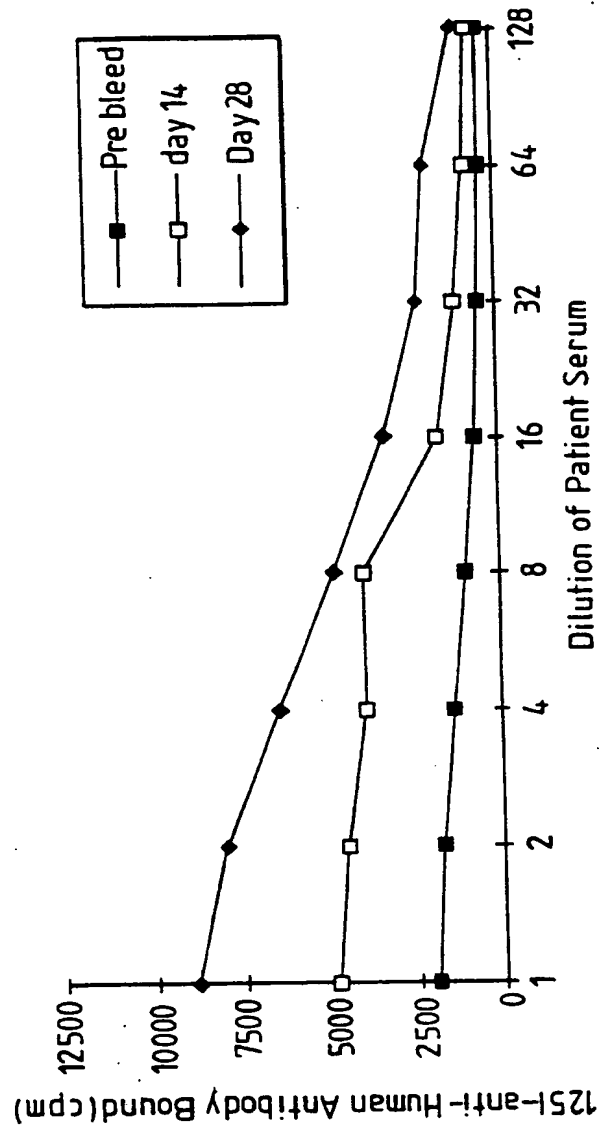


Fig.11B.



10/12

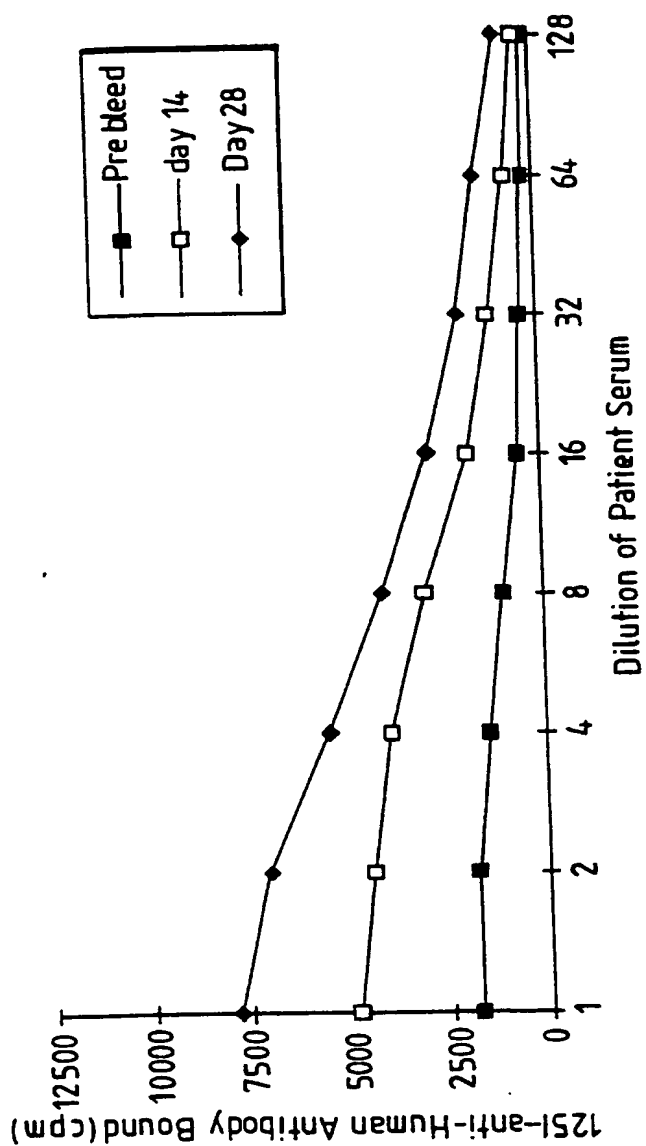


Fig.11C.

11/12

Fig.12A.

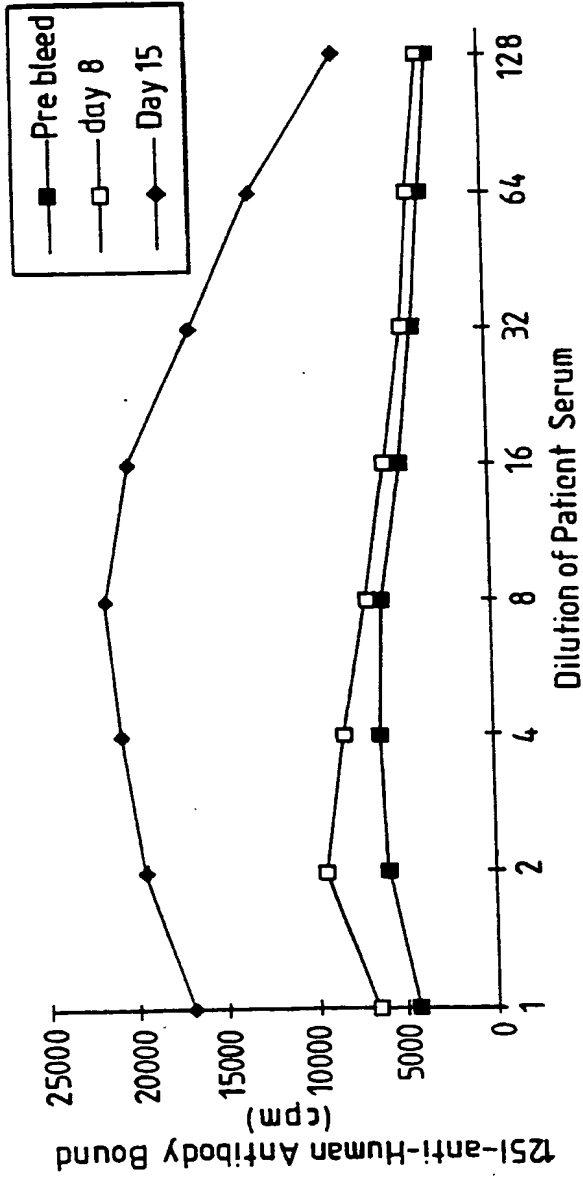
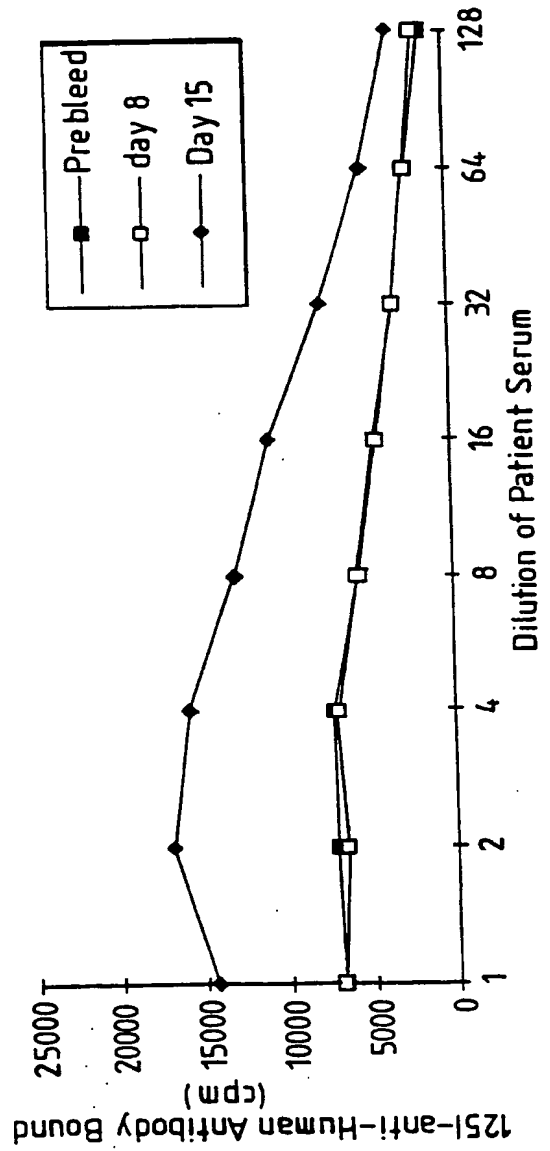


Fig.12B.



12/12

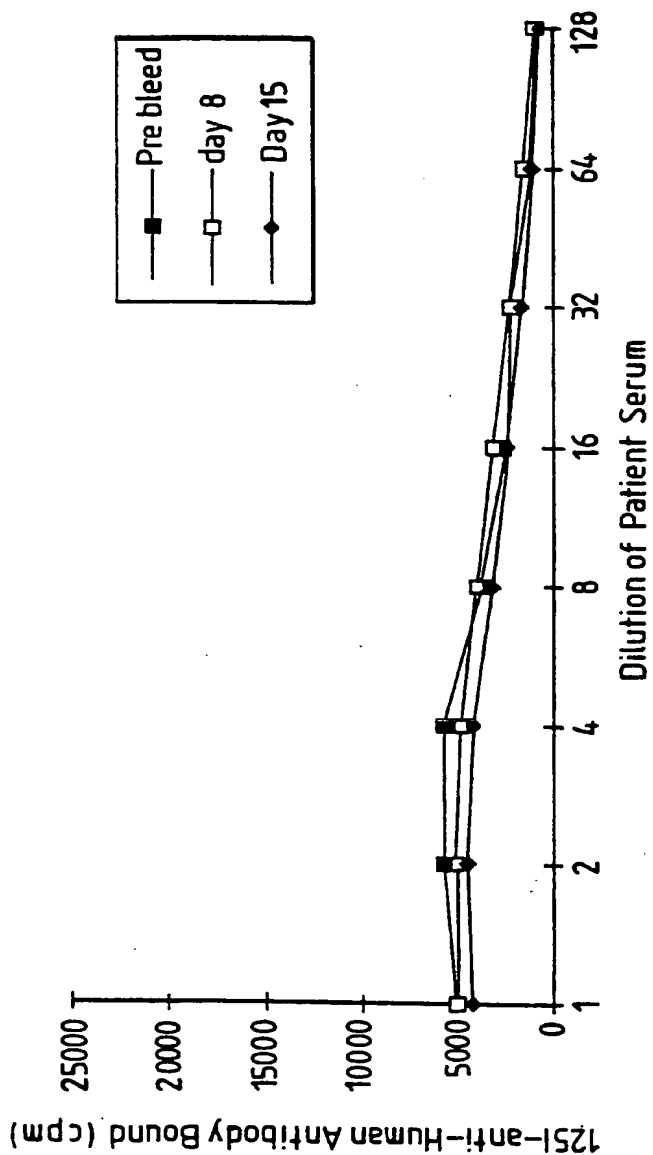


Fig.12C.

INTERNATIONAL SEARCH REPORT

Internal Application No.

PCT/GB 95/00118

A. CLASSIFICATION OF SUBJECT MATTER

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/28 A61K39/395 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BRITISH JOURNAL OF CANCER, vol.67, no.2, February 1993, LONDON, GB pages 247 - 253 H. MODJTAHEDI ET AL. 'The human EGF receptor as a target for cancer therapy: six new rat mAbs against the receptor on the breast carcinoma MDA-MB 468.' cited in the application see abstract</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1-10, 14-16</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- * 'A' document defining the general state of the art which is not considered to be of particular relevance
- * 'E' earlier document but published on or after the international filing date
- * 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * 'O' document referring to an oral disclosure, use, exhibition or other means
- * 'P' document published prior to the international filing date but later than the priority date claimed

- "T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&"** document member of the same patent family

Date of the actual completion of the international search

11 April 1995

Date of mailing of the international search report

02.05.1995

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 95/00118

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BRITISH JOURNAL OF CANCER, vol.67, no.2, February 1993, LONDON, GB pages 254 - 261 H. MODJTAHEDI ET AL. 'Immunotherapy of human tumour xenografts overexpressing the EGF receptor with rat antibodies that block growth factor-receptor interaction.' cited in the application see abstract</p> <p>---</p>	1-10, 14-16
X	<p>INTERNATIONAL JOURNAL OF ONCOLOGY, vol.3, no.2, August 1993, ATHENS, GREECE pages 237 - 243 H. MODJTAHEDI ET AL. 'The growth response of human tumour cell lines expressing the EGF receptor to treatment with EGF and/or Mabs that block ligand binding.' see abstract</p> <p>---</p>	1-10, 14-16
X	<p>CELL BIOPHYSICS, vol.22, no.1-3, January 1993, CLIFTON NJ, USA pages 129 - 146 H. MODJTAHEDI ET AL. 'Antitumor activity of combinations of antibodies directed against different epitopes on the extracellular domain of the human EGF receptor.' cited in the application see abstract see discussion</p> <p>---</p>	1-10, 14-16
X	<p>CANCER RESEARCH, vol.53, no.18, 15 September 1993, PHILADELPHIA PA, USA pages 4322 - 4328 Z. FAN ET AL. 'Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies.' cited in the application see abstract see discussion</p> <p>---</p>	1,3,4, 6-10, 14-16
X	<p>WO,A,92 15683 (MERCK PATENT GMBH) 17 September 1992 see examples see claims</p> <p>---</p>	1,3,4, 6-16
1 X	<p>US,A,4 943 533 (UNIVERSITY OF CALIFORNIA) 24 July 1990 see the whole document</p> <p>---</p> <p>--- -/--</p>	1,3,4, 6-10, 14-16

INTERNATIONAL SEARCH REPORT

Inter nal Application No

PCT/GB 95/00118

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.268, no.32, 15 November 1993, BALTIMORE MD, USA pages 23860 - 23867 K. CARRAWAY ET AL. 'Inhibition of epidermal growth factor receptor aggregation by an antibody directed against the epidermal growth factor receptor extracellular domain.' cited in the application see abstract -----	1,3,7
P,X	CANCER RESEARCH, vol.54, no.7, 1 April 1994, PHILADELPHIA PA, USA pages 1695 - 1701 H. MODJTAHEDI ET AL. 'Differentiation or immune destruction: Two pathways for therapy of sqamous cell carcinoma with antibodies to the epidermal growth factor receptor.' cited in the application see abstract -----	1-10, 14-16
P,X	INTERNATIONAL JOURNAL OF CANCER, vol.57, no.SUP8, 1994, GENEVA, SWITZERLAND pages 103 - 107 C. DEAN ET AL. 'Immunotherapy with antibodies to the EGF receptor.' see abstract -----	1-10, 14-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 95/00118

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9215683	17-09-92	AU-A- 1340392 CZ-A- 9203327 EP-A- 0531472 HU-A- 65687	06-10-92 16-02-94 17-03-93 28-07-94
US-A-4943533	24-07-90	NONE	

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.